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# Status on methicillin resistant and multiple drug resistant *Staphylococcus aureus* in fishes of Cochin and Mumbai coast, India

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**Abstract**

The present study was carried out to estimate the prevalence of Methicillin resistant and multiple Drug resistant (MDR) *S. aureus* in retail fishery outlets of Cochin and Mumbai coast of India. In total 252 *Staphylococcus aureus* isolates were obtained from 105 fish samples and further confirmed by polymerase chain reaction (PCR) using 16S rDNA region specific primer for *S. aureus*. In antibiogram analysis against 20 antimicrobial agents, highest resistant was found to erythromycin (31.14%), followed by azithromycin (25.79%) and clarithromycin (20.24%). One *S. aureus* isolate was resistant to methicillin, which was further confirmed by PCR using *mecA* gene primers specific for MRSA. The higher incidence of MDR *S. aureus* and the presence of MRSA in fish indicate high risk to public health.

**Key words**

Antibiogram, *mec*A, MDR, MRSA, *S. aureus*

## Introduction

*Staphylococcus aureus* is one of the major food-poisoning bacteria. However, now-a-days its multiple resistance to various types of antibiotics is a major concern than food poisoning outbreak (EFSA, 2010). The widespread use of antibiotics has evolved the emergence of multi-drug resistant strains that makes its eradication more difficult and its incidences are also increasing steadily. Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the multiple drug resistant *S. aureus* which has been causing serious nosocomial infection throughout the world for the past two decades (Nandalal and Somashekar, 2007). Methicillin was introduced as new β-lactam antibiotic in 1950 to overcome penicillin resistance *S. aureus*. A decade later in 1961, MRSA was reported in United Kingdom (Jevons, 1961), it is resistant to most recent β-lactam antibiotics including penicillin, cephalosporins, carbapenems and their derivatives (Katayama *et al.,* 2000). The resistant *S. aureus* produces an enzyme called penicilinase that alters penicillin binding proteins (PBPs) which cleave β-lactam ring of penicillin. Hence, the antibiotics such as penicillin G, methicillin, naficillin, oxacillin, cloxacillin, di-cloxacillin and fluciloxacillin have become ineffective. Owing to the ability of the bacteria to evolve resistance against various antibiotics, the incidence of MRSA has increased from 2% in 1974 to 64% in 2004 (Emily *et al.,* 2010). MRSA is classified into Hospital Associated MRSA (HA-MRSA) and Community Associated MRSA (CA-MRSA) (Doyle *et al.,* 2011). MRSA is highly prevalent in clinical samples. Limited reports are available on the prevalence of MRSA in meat sample and its prevalence in fish. Since seafood is handled by various people before reaching consumers, the possibility of occurrence of multiple drug resistant (MDR) and MRSA in sea food is relatively high. Under this context, the present study was designed to find MDR and MRSA in sea food samples collected from Cochin and Mumbai coast of India.

## Materials and Methods

Marine fish samples *viz., Pampus argenteus, Tylosurus crocodiles, Loligo duvauceli, Mugil cephalus, Chirocentrus dorab, Rastrelliger kanagurta, Nemipterus japonicas, Sardinella longiceps, Epinephelus sp., Cynoglossus sp., Arius sp., Himantura sp.* and *Scomberomorus sp*. were collected in sterile

polyethylene bags from commercial fishery outlets of Ernakulum, Alappuzha and Kottayam districts of Kerala and Vashi, Navi Mumbai regions in Maharashtra, India

10 g of skin and muscle tissue of fish sample was added to 90 ml of Brain Heart Infusion broth (BHIB) (Difco) supplemented with 6.5% NaCl (Karabiber *et al.*, 2009), then the samples were blended using a stomacher blender (Seward, UK). Subsequently, blended materials were serially diluted and plated onto Baird Parker (BP) agar (Difco, #276840). Two to three numbers of typical colonies (black colonies surrounded by thin white precipitation followed with a halo zone) were isolated and confirmed by coagulase production and amplification of 16S rDNA specific to *S. aureus* by polymerase chain reaction.

Antibiogram was performed by spreading 0.5% Mc Farland standard concentration of bacterial culture over 200 mm diameter petri plates pre-set with Muller Hinton agar containing 2% salt. Then 20 antibiotics discs (Himedia, #IC002) were laid over the plates and incubated in inverted condition for 24 hr to 48 hr. Standard MRSA strain (ATCC 333591) and *Methicillin sensitive Staphylococcus aureus* (MSSA) (ATCC 25923) were kept as control. Phenotypic oxacillin resistance of *S. aureus* was further confirmed by streaking over Mueller-Hinton agar (Difco) containing 2% NaCl and 6 µg of oxacillin per ml.

**Amplification of 16S rDNA and *mecA* gene :** DNA from *S. aureus* strains were isolated using GenElute bacterial genomic kit (Sigma, # NA2110). Initial cell lysis was carried out using Lysozyme (Sigma, # L6876) and Lysostaphin (Sigma, # L7386), followed by DNA isolation (Sambrook and Russell, 2001). Duplex PCR was carried out for all isolates for confirmation of *S. aureus* and MRSA. *MecA* gene (methicillin resistance gene) and 16s*rDNA* (*Staphylococcus aureus s*pecific region) primers were used for PCR (Zhang *et al.,* 2004). The primer sequences such as SAF (5' AAC TCT GTT ATT AGG GAA GAA CA 3') and SAR (5' CCA CCT TCCTCC GGT TTG TCA CC 3') are specific to *16S rDNA*, which amplifies the *S. aureus* specific region. *MecAF* (5' GTA GAA ATG ACT GAA CGTCCG ATA A 3') and *mecAR* (5' CCA ATT CCA CAT TGT TTC GGT CTAA 3') are specific for *mecA gene.*

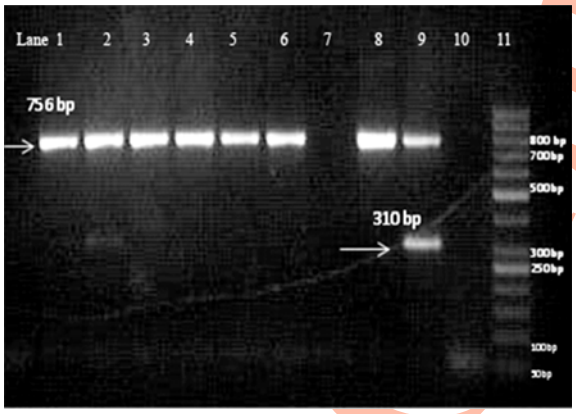


**Fig. 1 :** Amplification of mec A gene (310 bp) and 16s *rDNA* (756 bp) from *Staphylococcus aureus* isolates from fish; Lane 1 to 6 and 8: amplicon size 756bp for *Staphylococcus aureus* confirmation, Lane 9: amplicon size 756bp for *Staphylococcus aureus* and amplicon size 301bp for the *mec A* gene, Lane 7 is negative control, Lane 10: Blank, Lane 11: Ladder (fermentas)

Two µl of DNA was added to 23 µl of PCR reaction mixture containing 50mM KCl, 20mM Tris-HCl (pH 8.4), 2.5mM $MgCl_2$, 0.2mM each deoxynucleoside triphosphate dNTPs mix (Fermentas), 0.12 picoM each *16S rDNA* and *mecA* primers (Sigma aldrich), 1.0 U of Taq DNA polymerase (Fermentas). Amplification was performed by using veriti thermal cycler (Applied Biosystems, Foster City, Calif.). The procedure for amplification was as follows, an initial denaturation step at 94°C for 5 min; 10 cycles of 94°C for 40 sec, 58°C for 40 sec, and 72°C for 1 min; 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. PCR products were electrophoresed on 1.5% agarose gel. A positive result was inferred by detection of 310-bp band, which is part of *mecA* gene and 756-bp band specific for 16s*rDNA* (Fig. 1).

## Results and Discussion

Among 105 seafood samples collected, 98 were found positive for *S. aureus. i.e.,* 93% of fish samples were contaminated with *S. aureus.* Totally, 252 *S. aureus* strains were isolated from 105 samples and all 252 isolates were subjected to antibiogram against 20 antibiotics. The antibiogram result revealed that most of the *S. aureus* were resistant to erythromycin followed by azithromycin, clarithromycin, penicillin G and ampicillin etc., (Table 1). The interesting observation was that first five antibiotics except penicillin were commonly used for the treatment of upper respiratory tract infection. First three antibiotics viz. erythromycin, azithromycin and clarithromycin belonged to Macrolid antibiotic class (table 2). Few strains were resistant to gentamicin, amikacin, ofloxacin, tetracycline, co-trimoxazole and Novobiocin. Only one strain of *S. aureus* was resistant to methicillin, oxacillin and cephalothin. All strains were susceptible to the recent generation of antibiotics such as vancomycin, teicoplanin, linezolid and clindamycin. Even though chloramphenicol is an older generation drug, all strains were found to be susceptible; this may be attributed to the rare application of chloramphenicol in disease treatment because of its toxicity. The high level of resistance was observed for macrolids classes followed by β lactam, aminoglycoside, quinolone, aminocoumarin and sulpha classes. No resistance was observed in case of glycopeptides, lincosamines and chloramphenicol (Table 2).

*S. aureus* is not a normal inhabitant of marine environment. But, it's presence in seafood mostly occurs through

**Table 1 :** Resistant pattern for *S. aureus*

| Name of the antibiotics | No. of resistant isolates | % of resistant isolates | 15B | MRSA (ATCC 333591) | MSSA (ATCC 25923) |
|---|---|---|---|---|---|
| Erythromycin (ERY) | 81 | 32.14 | Susceptible | Resistant | Susceptible |
| Azithromycin (AZ) | 65 | 25.79 | Susceptible | Resistant | Susceptible |
| Clarithromycin (CLA) | 51 | 20.24 | Susceptible | Resistant | Susceptible |
| Penicillin G (PEN) | 62 | 24.60 | Resistant | Resistant | Susceptible |
| Ampicillin (AMP) | 72 | 28.57 | Resistant | Resistant | Susceptible |
| Amoxyclav (AMC) | 59 | 23.41 | Susceptible | Resistant | Susceptible |
| Oxacillin (CLA) | 1 | 0.40 | Resistant | Resistant | Susceptible |
| Methicillin (MET) | 1 | 0.40 | Resistant | Resistant | Susceptible |
| Cephalothin (CEP) | 1 | 0.40 | Resistant | Resistant | Susceptible |
| Gentamicin | 26 | 10.32 | Susceptible | Susceptible | Susceptible |
| Amikacin (AK) | 14 | 5.56 | Susceptible | Susceptible | Susceptible |
| Ofloxacin (OFN) | 12 | 4.76 | Susceptible | Susceptible | Susceptible |
| Tetracycline (TET) | 8 | 3.17 | Susceptible | Susceptible | Susceptible |
| Co-trimoxazole (COT) | 12 | 4.76 | Susceptible | Susceptible | Susceptible |
| Novobiocin (NB) | 4 | 1.59 | Susceptible | Susceptible | Susceptible |
| Vancomycin (VAN) | 0 | 0.00 | Susceptible | Susceptible | Susceptible |
| Teicoplanin (TEI) | 0 | 0.00 | Susceptible | Susceptible | Susceptible |
| Linezolid (LZ) | 0 | 0.00 | Susceptible | Susceptible | Susceptible |
| Chloramphenicol (CLO) | 0 | 0.00 | Susceptible | Susceptible | Susceptible |
| Clindamycin (CLN) | 0 | 0.00 | Susceptible | Susceptible | Susceptible |

Total number of isolates are 252; (MRSA: Methicillin Resistant *Staphylococcus aureus*); (MSSA: Methicillin Susceptable *Staphylococcus aureus)*

postharvest handling. In most of the Indian landing centres, fish are often usually cleaned with contaminated sea water before being sold; this would be a major source of contamination for *S. aureus*. Soge *et al. (*2009) and Zhang *et al*. (2013) reported that most of the beaches and coastal regions of the world are contaminated with MDR bacteria. Hunter *et al. (*2010) also reported that MDR bacteria can be transferred from humans to the environment and animals. Next source of *S. aureus* contaminations comes through the storage tank located in fishing vessel and also from the utensils used for carrying fish. In Fortaleza (Brazil), 60% of the fish handlers were harbouring MDR *S. aureus* and it was isolated both from fish handlers and food utensils (Albuquerque *et al.,* 2000). In the world, 60% of the fishermen population harbours *S. aureus* (Evangelista-Barreto *et al.,* 2003) about 50% and 20 % of the human population harbours *S. aureus* in skin and intestine respectively (Doyle *et al.,* 2001). These reports suggest that *S. aureus* has arrived through fish handlers; likewise MRSA would have come through fish handlers only.

MRSA is frequently isolated from clinical samples due to its high prevalence in hospital environment. In case of fish sample, the prevalence study has not yet been done. But, in the present study, out of 105 samples, MRSA were found in one sample only; this is due to less prevalence of *S. aureus* in commercial fish market. In Korea preliminary study was conducted for detecting MRSA in fish samples by Rhee and Woo (2010). They isolated two MRSA strains from fish and raw meat which were found to be enterotoxigenic and resistant to β-lactam antibiotics. The result suggests that MRSA is not only present in clinical samples but it exists in food materials such as meat and fish. Still limited reports are available regarding MRSA in meat and other animals. But, in fish it is unknown. So, the prevalence of MRSA in fish still needs to be explored. Moreover, arrival of MRSA is either of hospital origin MRSA(HA-MRSA) or Community associated MRSA (CA-MRSA). Infected handlers may be the reason for spread of MRSA. But, in the present study, isolated MRSA seems to be a CA-MRSA strain because, HA-MRSA strains are usually resistant to most of the antibiotics, eg. Standard strain (ATCC 333591) was resistant to penicillin, erythromycin, azithromycin and clarithromycin (Table 1). However, the MRSA strain isolated (15B) in this study was resistant only to oxacillin, cephalothin and penicillin; but it was susceptible to tetracyclin and clindamycin. Raygada *et al. (*2009) reported that HA-MRSA responds to a newer class of antibiotics only but the CA-MRSA is very much susceptible to Sulpha (Co–trimaoxazole), tetracycline and Clindamycin; Likewise, MRSA isolate, in the present study, was also susceptible to the same above said antibiotics.

Duplex PCR is used for simultaneous detection of *S. aureus* and MRSA. All the isolates were subjected to PCR for confirmation of *S. aureus*. Primes specific to *S. aureus* are in 16S rDNA region and used for amplification of product size 756bp (Fig. 1). Amplification of 310bp in agarose gel indicates the product of *mecA* gene which is specific for MRSA.

**Table 2 :** Resistant level of the isolates with different classes of antibiotics

| Antibiotic classes | No. of Resistant strain | % of resistant isolates |
|---|---|---|
| Macrolids (ERY, AZ & CLA) | 81 | 32.14 |
| Â lactam ( PEN, AMP, OXA, MET, AMC & CEP) | 77 | 30.56 |
| Aminiglycosides (GEN & AK) | 17 | 6.75 |
| Quiniolone ( OFN) | 11 | 4.37 |
| Amino coumarion (NOV) | 4 | 1.59 |
| Sulpha (COT) | 5 | 1.98 |
| Glycopeptides (VAN & TEI) | 0 | 0.00 |
| Lincosamines (LZ) | 0 | 0.00 |
| Chlorampenicol (CLO) | 0 | 0.00 |

In the present study, 32.14% of *S. aureus* showed MDR to various antibiotic classes. In case of Indian hospitals MDR level of *S. aureus* went upto 70% (Arora *et al.,* 2010). Which indicates that MDR percentage level of *S. aureus* of fish market origin is lesser resistant than of clinical origin. Even though *S. aureus* isolates of fish market are lesser resistant when compared with that of clinical origin, still they are more threatening to consumers. More over the incidence of MDR *S. aureus* is more than 30%, which indicates that fish markets are also an important place for transfer of MDR *S. aureus*.

In general opinion, MRSA is mostly restricted to clinical samples, the present study revealed that it is also prevalent in fish market. The study also revealed the occurrence of high levels of MDR strains from fishes off the coastal region. This dangerous indication assesses the status of unhealthy handling of seafood by fishermen. Hence, all fish handlers should be made aware on the importance of personal hygiene and hygienic handling practices for ensuring the supply of safe seafood. A series of detailed guidelines published by the UK regulatory control of MRSA can be followed to decrease transboundary diseases through export and import.

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