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Antibiotic Resistance Pattern of *Staphylococcus aureus* Isolated from Seafood

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Abstract

Staphylococcus aureus is a pathogen known to cause hospital, community acquired and foodborne illness with varying susceptibility to antibiotics. The selective pressure due to the use of antibiotics along with horizontal and vertical gene transfer has resulted in the evolution of multidrug resistant *S. aureus*. The purpose of the study was to determine the antibiotic resistance pattern in *S. aureus* isolated from seafood of Kerala, India. One hundred and thirty three *S. aureus* isolates obtained during 2012 to 2015 were included in this study. Antibiotic susceptibility testing was performed with a set of 20 antibiotics representing eleven classes of antibiotics by standard disk diffusion assay. The study revealed that 90.9% of the *S. aureus* isolates were resistant to at least one class of antibiotics. Resistance was found among 33.8, 27.8, 17.3, 6.8, 3.8, 0.7 and 0.7% isolates to one, two, three, four, five, six and eight classes of antibiotics respectively. Multidrug resistance was found in 29.3% of the *S. aureus* isolates with resistance to antibiotics ranging from 3 to 8 classes of antibiotics. The study reveals that *S. aureus* isolates were sensitive to aminoglycosides and phenicols.

Keywords: *Staphylococcus aureus*, multidrug resistance, Antimicrobial susceptibility testing, CLSI

Introduction

S. aureus is considered as an organism of major public health concern worldwide. The organism is capable of producing various diseases of skin and soft tissues; and in some cases can produce, sepsis, osteomyelitis and pneumonia (EFSA, 2010; Vázquez-Sánchez et al., 2012; Crago et al., 2012). *S. aureus* has multiple pathways for adapting to various environmental conditions and gains resistance to many antibiotics either through mutations or rearrangements within the Staphylococcal genome, or by the acquisition of resistance determinants (McCallum et al., 2010). The increasing use of antibiotics along with the spread of resistance genes and bacteria has resulted in the evolution of bacterial resistance (Levy, 2002). History of antibiotic resistance in *S. aureus* was described way back in 1940's when Kirby first demonstrated penicillin-resistant *S. aureus.* The introduction of methicillin in 1961 was immediately followed by reports of methicillin resistance (Jevon, 1961). Subsequently fluoro-quinolones (Quinolone) resistance (Lowy, 2003), vancomycin intermediate-resistant *S. aureus* (VISA) (Hiramatsu et al., 1997), linezolid-resistant *S. aureus* (Tsiodras et al., 2001) and Daptomycin resistance MRSA were reported (Mangili et al., 2005). Currently, MRSA strains are reported to cause nosocomial infection with a prevalence ranging from 33 to 55% in US hospitals (Appelbaum, 2006).

Seafood products are of high nutritional value (Amagliani et al., 2012) and are often associated with foodborne pathogens (Onmaz et al., 2015). *S. aureus* is not a native flora of fish and fishery products (Huss, 1988). Contamination of seafood results from improper handling, storage and cross contamination (Tranter 1990; Huang et al., 2001; Simon & Sanjeev, 2007; Waters et al., 2011). Antibiotic-resistant strains of *S. aureus* have been isolated from food animals, meat, milk, dairy products and fishery products (Lee, 2003; Normanno et al., 2007; Pereira et al., 2009; Beleneva, 2011; Grema et al., 2015). Studies on the antibiotic resistance pattern of *S. aureus* isolated from seafood in India is limited. Multidrug-resistant Staphylococci are a growing problem for many health care institutions. The incidence of drug-resistant pathogens differs greatly between countries according to

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differences in the usage of antibiotics. Evolution of antibiotic resistance is a continuous process in *S. aureus* which depends on antibiotic usage. The aim of the study was to determine changes in antibiotic resistance pattern of *S. aureus* isolated from seafood from Kerala, India.

Materials and Methods

In this study, a total of 133 *S. aureus* isolated from raw seafood such as finfish (113 isolates) and shellfish (20 isolates) during 2012 to 2015 from retail fish markets of Kerala were included. *S. aureus* isolates from retail fish markets included in this study were Edavanakkad (26), Thevara (26), Polakkandam (29), Varapuzha (26), Njarakkal (3), Malipuram (2) and North Paravur (5) of Ernakulam district; Kottayam (2) of Kottayam district and Cherthala (14) of Alapuzha district. Cultures were maintained in -80ºC as glycerol stocks until use.

Molecular confirmation of *S. aureus* was done by PCR amplification (Master mix 1X, Thermoscientific, USA) as desribed by Strommenger et al. (2003) in a 25 µl reaction volume. The reaction mixture contained approximately 10 ng of template DNA (cell lysis method), 2.5 pmol of each primer Sau1 F AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG, Sau2 R CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA (Sigma Aldrich, India). The thermal

Table 1. Antibiotic resistance pattern of *S. aureus* from seafood

No. of Antibiotic classes	Antibiotic resitance Profile	No. of Strains	Antibiotic resistance (%)
1	B	41	33.8
	G	2	
	L	1	
	M	1	
2	B + (M/T/Q/G/O/FPI)	35	27.8
	M + Q	1	
	G + T	1	
3	B + M + (T/L/Q/A/G)	17	17.3
	B + O + (L/A)	5	
	B + Q + G	1	
4	B + M + FPI + Q	1	6.8
	B + M + O + (L/T)	2	
	B + M + G + (O/T)	2	
	B + M + L + A	1	
	B + G + ASB + P	1	
	B + G + ASB + Q	1	
5	B + M + ASB + G + (L/Q/A)	3	3.8
	B + M + Q + G + O	1	
	B + M + ASB + FPI + P	1	
6	B + M + ASB + L + A + T	1	0.7
8	B + M + ASB + L + A + T + G + O	1	0.7

Note: Beta-lactams antibiotics (B): Penicillin (P), Ampicillin (AMP), Amoxyclav (AMC); Anti-Staphylococcal Beta-lactams (ASB): Methicillin (MET), Oxacillin (OX); Cephalosporins: Cephalothin (CEP); Glycopeptides (G): Teicoplanin (TEI), Vancomycin (VA); Aminoglycosides (A): Gentamicin (GEN), Amikacin (AK); Tetracycline (T): Tetracycline (TE); Quinolones (Q): Ofloxacin (OF); Oxazolidinones (O): Linezolid (LZ); Folate pathway inhibitor (FPI): Co-Trimoxazole (COT); Macrolides (M): Erythromycin (E), Azithromycin (AZM), Clarithromycin (CLR); Lincosamides (L): Clindamycin (CD); Phenicols (P): Chloramphenicol (C).

profile followed was, initial denaturation at 94°C for 3 min followed by 30 cycles of amplification; 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s and final extension of 4 min. PCR products were analyzed on a 1.5% agarose gel.

The isolates were characterized for antibiotic resistance pattern by antimicrobial susceptibility testing (AST) with an array of 20 antibiotics (Icosa GI-Plus) arranged in circular pattern separated at least 24 mm apart (Himedia, India. Cat.No.IC002) by standard disk diffusion assay as per Bauer et al. (1966) and break point interpretation were performed as per the manual CLSI (2007; 2014). The antibiotics panel included were beta-lactams antibiotics; (Penicillin (P) 10 U, Ampicillin (AMP) 10 µg, Amoxyclav (AMC) 30 µg), Anti-Staphylococcal beta-lactams (Methicillin (MET) 5 µg, Oxacillin (OX) 1 µg) and cephalosporins (Cephalothin (CEP) 30 µg); Glycopeptides (Teicoplanin (TEI) 10 µg, Vancomycin (VA) 30 µg); Aminoglycosides (Gentamicin (GEN) 10 µg, Amikacin (AK) 30 µg); Tetracycline (Tetracycline (TE) 30 µg); Quinolones (Ofloxacin (OF) 5 µg); Oxazolidinones (Linezolid (LZ) 30 µg); Folate pathway inhibitors (trimethoprim/sulfamethoxazole Co-Trimoxazole (COT) 25 µg); Macrolides (Erythromycin (E) 15 µg, Azithromycin (AZM) 15 µg, Clarithromycin (CLR) 15 µg); Lincosamides (Clindamycin (CD) 2 µg); Phenicols (Chloramphenicol (C) 30 µg) and Aminocoumorins (Novobiocin (NV) 5 µg). Cultures grown overnight in Trypticase Soy Broth or young culture from TSA slants adjusted to 0.5 McFarland turbidity standard were swabbed onto the Muller–Hinton agar medium; antibiotic disc were placed aseptically and incubated at 35°C for 16 to 18 h. *S. aureus* (ATCC 29213), *S. aureus* (ATCC 43300) *Escherichia coli* (ATCC 25922) and *E. coli* (ATCC 35218) were used as control strains.

Results and Discussions

PCR targeting conserved region of *S. aureus* using *sau* primer gave an amplicon of 100 bp which confirmed the identity of the organism (Fig. 1). Antibiotic susceptibility testing of *S. aureus* isolates revealed that all the isolates were novobiocin susceptible indicating that all the isolates were *S. aureus* (Novobiocin susceptible coagulase positive Staphylococci). In this study, only twelve isolates (9%) had antibiotic susceptible phenotypes, while remaining 90.9% of strains showed antibiotic resistance to atleast one antibiotic. Order of antibiotic resistances observed among the *S. aureus* isolates in this study were beta lactam penicillins (AMP 83.4%, P 78.9%, AMC 75.9%) > Macrolides (E 30.8%, CLR 27.0%, AZM 24.06%) > Quinolones (OF 11.2%) > Oxazolidinones (LZ 10.2%) > Glycopeptides (VA 9.7%, TEI 5.2%) > Lincosamides (CD 9.0%) > Cephalosporin (CEP 7.5%) > Tetracycline (TE 5.2%) > Aminoglycosides (GEN & AK 3.0%) > Folate pathway inhibitors (2.2%) > phenicols (2.2%) (Fig. 2). Analysis of the antibiotic resistance pattern revealed that 39 isolates were resistant to three or more antibiotics. Magiorakos et al. (2012) has defined multidrug resistant *S. aureus* as a strain that acquired non-susceptibility to at least one agent in three or more antimicrobial categories. The phenotypic characteristics of MDR *S. aureus* isolated from seafood that showed resistance to atleast one drug are presented in Table 1. One MDR isolate was found in Kottayam retail market, where as in Alappuzha district five MDR isolate were present among 14 isolates screened with resistance to 3-4 classes of antibiotics. Thirty three MDR isolates were from Ernakulam district with resistance ranging from 3-8 classes of antibiotics. Edavanakkad market contributed eleven MDR *S. aureus* isolates with resistance of 8 classes resistance of antibiotics. While ten MDR *S. aureus* were from Polakkandam retail market with resistance to 6 classes of antibiotics. The number of MDR *S. aureus* isolates from Thevara, Kochi and Varapuzha retail markets were five and seven respectively with resistance upto five classes of antibiotics. A study conducted by Jeshina & Surekha (2009) from infected pus of human patients in Calicut region of Kerala with 70 *S. aureus* strains have demonstrated that most of isolated in their studies were MRSA with multidrug resistant phenotype. The order of resistance observed in their study was ampicillin (100%), oxacillin (100%), ciprofloxacin (81.4%), co-trimoxazole (71.4%), mupirocin (68.5%), fusidic acid (58.5%), ofloxacin (51.4%), erythromycin (45.7%), tetracycline (44.2%), ceftriaxone (42.8%), chloramphenicol (24.2%) and Vancomycin (2.8%). All the strains were susceptible to gentamicin. The change in the resistance pattern of gentamicin resistance to ciprofloxacin resistance was best explained with the change in trend of using antibiotics in the therapy.

In another study conducted at Jalandhar city, India by Kumar et al. (2015), the antibiotic resistance pattern of *S. aureus* isolated from food, nasal swab, waste water and paper currency determined against 23 antibiotics revealed that 130 isolates from

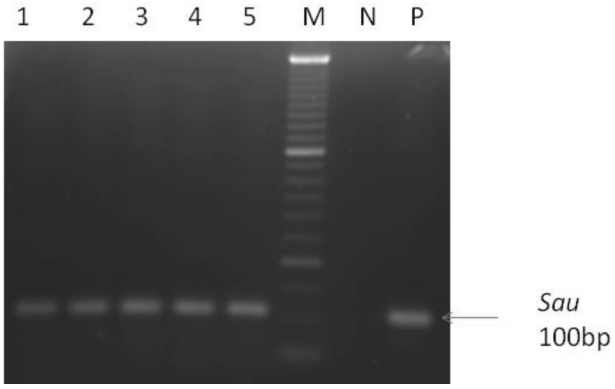

Fig. 1. PCR for confirmation of *S. aureus*

Lane 1 to 5: Species specific *Sau* positive *S. aureus*
Lane M: Molecular weight market 50 bp
Lane N: Negative control
Lane P: Positive control *S. aureus* ATCC 43300

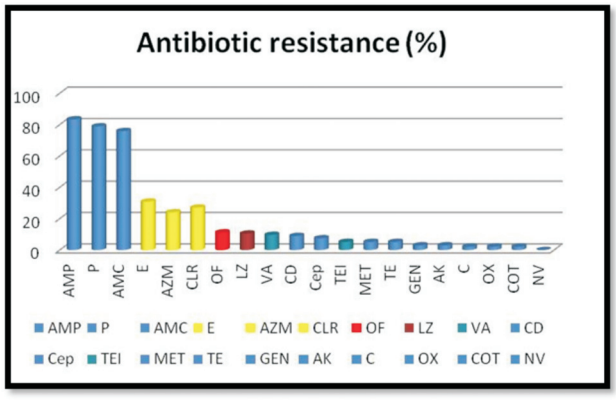

Fig. 2. Antibiotic resistance in *S. aureus* (2012-2015)

different sources showed multidrug resistance. The order of antibiotic resistance were Penicillin (91.39%) > Methicillin (85.51 %) > Cefopodoxime (83.54%) > Clindamycin (71.24%%) > Oxacillin (64.61%) > Tetracycline (61.7 %) > Doxycycline (58.55%) > Amoxycillin-Clavulanic acid (52.8%) > Vancomycin (51.34%) > Carbapenam (50.5%) > Ceftrazoline (47.97%) > Cephalothin (44.65%) > Azithromycin (42.09%) > Nitrofurantoin (37.43%) > Gatifloxacin (33.93%) > Tobramycin (26.25%) > Piperacillin-tazobactum (26.15%) > Ciprofloxacin (25.34%) > Norfloxacin (22.72%) > Cefotaxime (22.62%) > Levofloxacin (20.73%) > Meropenam (12.54%) > Amikacin (11.13%) indicating higher resistance was found to beta lactam and lowest for aminoglycosides.

The present study was based on *S. aureus* isolated from seafood of retail markets of Kerala, with resistance to beta-lactam (75-83%) and macrolides (24-30%) antibiotics were higher, compared to other classes of antibiotics with resistance lower than 15%. The resistance to aminoglycosides, phenicols and folate pathway inhibitor were less than 3%. This could be because of decreased use of aminoglycosides, folate pathway inhibitors and phenicols in therapy as described by Jeshina & Surekha (2009).

A study by Albuquerque et al. (2007) on antibiotic resistance in *S. aureus* from fish markets and workers revealed the prevalence of multidrug resistant *S. aureus* isolates at 45.5 and 40% respectively. Sanjeev & Mahadeva Iyer (1988) reported antibiotic resistance pattern in one hundred and twenty two *S. aureus* isolates from fish processing factory workers of Cochin, Kerala and higher resistance were observed against ampicillin (64.75%), pencillin (59.84%) and Visnuvinayagam et al. (2015) has investigated antibiotic resistance pattern of 252 of *S. aureus* strains from 105 seafood samples of Cochin and Mumbai which revealed that order of resistance was macrolides (20-32.1%) followed by β-lactams (23.4-28.5) aminoglycosides (5-10%), ofloxacin (4.7%), cotrimoxazole (4.7%) and tetracycline (3.1%). No resistance was observed in case of glycopeptides, lincosamines and chloramphenicol. Multidrug resistance was found among 32.14% of the *S. aureus* tested. Whereas the present study with *S. aureus* isolated from fish retail markets of Kerala revealed multidrug resistance in 29.3% of the isolates with resistance against glycopeptide, lincosamide, chloramphenicol at 5.2-9.7%, 9 and 2.2% respectevely.

In the present study even though reduced susceptibility to methicillin and oxacillin were found in 5.2 and 2.2% of the isolates respectively, none of them showed defined methicillin resistance characteristics when tested in oxacillin agar screen method and cefoxitin disc diffusion assay which clearly corroborates with the guidelines of (CLSI 2007 to 2014). These results indicates the need to use cefoxitin as surrogate marker for identification of methicillin resistant *S. aureus* instead of methicillin or oxacillin in disk diffusion assay.

The study also indicates a high prevalence (29.3%) of multidrug resistance in *S. aureus* isolated from seafood sold in Kerala. However no extensively-drug resistant (XDR) or pandrug-resistant (PDR)

strains were found. High diversity in the antibiotic resistance pattern could be noticed in the isolates tested. Lack of information on MDR *S. aureus* from rest of India, suggest surveillance on MDR *S. aureus* in seafood and environment. Determination of antibiotic resistance pattern in a defined geographical location over a period of time helps not only in assessing the evolution of resistance but also will act as a recknor for choosing antibiotics for treating community associated infections among food handlers in a defined region.

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