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Note

Prevalence of methicillin resistant *Staphylococcus aureus* in selected seafood markets and aquaculture farms in Kerala, south-west coast of India

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ABSTRACT

A study was carried out to understand the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in selected seafood commodities, market environment as well as in aquaculture farms in Kerala, along the south-west coast of India. Two hundred and thirty three samples comprising finfish, crustaceans, molluscs and fishery environmental samples from markets as well as aquaculture farms were collected and screened for the presence of MRSA. The *S. aureus* isolates obtained from the samples were checked for resistance to methicillin, oxacillin and cefoxitin by antimicrobial sensitivity test and further confirmed by oxacillin agar screening method. A multiplex PCR was employed targeting *mecA*, *nuc* and *16s rDNA* genes to differentiate *S. aureus* and MRSA from other staphylococci. It was found that 13.4% of the samples harboured MRSA while the overall prevalence of *S. aureus* was found to be 36.5%. This study has clearly indicated high prevalence of methicillin resistant *S. aureus* strains in the seafood as well as in environmental samples from seafood markets and aquaculture farms in Kerala. The prevalence of methicillin resistant strains of staphylococci in fish indicates high risk associated with fish handlers and fish farmers.

Keywords: *mecA* gene, Methicillin-resistant *Staphylococcus aureus,* MRSA, Prevalence, Seafood

Staphylococcus aureus, a well known pathogen of hospital acquired infection and food poisoning (Khan *et al.*, 2015), is a commensal organism of skin and mucous membrane of different species of animals as well as human beings and is not generally considered as the normal microflora of fish (Huss, 1988; Mc Carthy *et al.*, 2012). Remarkably, 20% of individuals are persistently and 30% are transiently colonised with *S. aureus* in the nose (Liu, 2009). When the methicillin sensitive *S. aureus* (MSSA) acquires the genes responsible for methicillin resistance, the organism causes higher morbidity and mortality in humans with prolonged period of hospitalisation and are called as methicillin resistant *S. aureus* (MRSA) (Datta and Huang, 2008). There are a few reports on the prevalence of *S. aureus* in fish and fishery products from local retail markets and imported samples (Ayulo *et al.*, 1994; Simon and Sanjeev, 2007; Saito *et al.*, 2011; Bujjamma and Padmavathi, 2015; Obaidat *et al.,* 2015). MRSA and methicillin resistant coagulase negative staphylococci (MR-CoNS) are recognised as zoonotic multidrug resistant pathogens causing hospital and community acquired infections in humans as well as infections in animals (Dahms *et al.*, 2014). Resistance to methicillin is determined by *mecA*, encoded for low affinity penicillin binding protein PBP2A (Beck *et al.,* 1986). Non-*mecA* mediated methicillin resistance in *S. aureus* may be due to excess production of beta-lactamase resulting in low level resistance to oxacillin called as borderline oxacillin resistant *Staphylococcus aureus* (BORSA) (Maalej *et al.,* 2012) or due to presence of a novel *mecA* gene homologue (*mecA*LGA251 or *mecC)* causing resistance to beta-lactam antibiotics (Garcia-Alvarez *et al.*, 2011). The prevalence of MRSA in fish was first reported from Malaysia in farmed tilapia (Atyah *et al.*, 2010). Visnuvinayagam *et al*. (2015,) and Kumar *et al.* (2016) reported on presence of MRSA from Indian seafood. The present study was conducted to investigate the prevalence of methicillin resistant *S. aureus* in seafood markets and aquaculture environments from selected locations along the south-west coast of India.

A total of 233 samples comprising finfishes (n=137), crustaceans (n=31), molluscs (n=26) and environmental samples (n=39) were collected during July 2012 to April 2015 from selected retail markets and aquaculture farms covering three districts along the south-west coast of India *viz.,* Ernakulam, Kottayam and Alappuzha. Environmental samples comprised water, swabs and ice from retail markets as well as water and sediment from aquaculture farms. Samples were transported to the laboratory in chilled condition and processed as per the standard protocol of United States Food and Drugs Administration (USFDA) for isolation of *S. aureus* in preprocessed food (Bennett and Lancette, 2001), with slight modifications. Ten gram of each sample was transferred to Tryptic soy

broth (TSB) (BD BBL & Difco, USA) supplemented with NaCl (10%) and sodium pyruvate (1%) and incubated at 35°C overnight. Serially diluted, overnight enriched sample (0.5 ml each) was inoculated by spread plating onto preset Baird-Parker agar base (BPA) (BD Difco, USA) supplemented with egg yolk potassium tellurite enrichment (BD BBL & Difco, USA). The plates were incubated at 35°C for 36 to 48 h. Additionally the enriched samples were plated onto BD Chromagar MRSA II (BD BBL & Difco, USA) and Oxacillin resistance agar screen base supplemented with ORSAB supplement (Oxoid, UK). The characteristic convex shiny black colonies surrounded by narrow white margin and clear zone from BPA plates; mauve colony on BD chromagar II; deep blue colony from ORSAB were regarded as presumptive MRSA. These suspected colonies were confirmed as *S. aureus* by biochemical tests (Bennett and Lancette, 2001) and the isolates were maintained in tryptic soy agar slants supplemented with 6% NaCl.

The isolates confirmed as *S. aureus* were subjected to standard antimicrobial susceptibility tests by disk diffusion assay (CLSI, 2012; 2014) with oxacillin (1µg), methicillin (5µg) and cefoxitin (30µg). Turbidity of overnight grown cultures in TSB were adjusted to 0.5 McFarland Unit and spread plated onto Mueller-Hinton agar (BD Difco, USA) and incubated at 35°C for 16 to 24 h. The results of susceptibility testing were interpreted as per CLSI (2014).

The isolates which showed reduced susceptibility to either or both oxacillin (1µg) or methicillin (5 µg) or cefoxitin (30 µg) were confirmed phenotypically for methicillin resistance by oxacillin agar screening method [Mueller-Hinton agar + oxacillin 6 µg ml^{-1} (Sigma) + 4% NaCl] as per CLSI (2012; 2014). *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213 were used as positive and negative control strains respectively in all the antimicrobial susceptibility testing and oxacillin resistance confirmation.

Rapid DNA extraction was performed with 1ml of overnight grown young culture in 500 µl of 1x TE buffer (pH 8.0), heated at 99°C for 10 min and cooled rapidly to -20°C until use. Multiplex PCR was performed as described by Zhang *et al.* (2004) in a Veriti Thermal Cycler (Thermofisher scientific, USA) with a 25 µl of PCR reaction mixture containing 3 µl of template DNA, 1x Taq buffer, 2.5mM $MgCl_2$, 200 µM dNTPs mix (Thermofisher scientific, USA), 0.12 µM of each primers (Staph756F 5-AAC TCT GTT ATT AGG GAA GAA CA-3; Staph750R 5-CCA CCT TCC TCC GGT TTG TCA CC-3 for *Staphylococcus* genus-specific 16S rRNA; *mecA* F 5-GTA GAA ATG ACT GAA CGT CCG ATA A-3 and *mecAR* 5-CCA ATT CCA CAT TGT TTC GGT CTA A-3 for methicillin resistance genes *mecA*); 0.04 µM of *nuc*F 5-GCG ATT GAT GGT GAT ACG GTT-3 and *nucR* 5-AGC CAA GCC TTG ACG AAC TAA AGC-3 for thermonuclease gene *nuc*; 0.65 µl of Taq polymerase 1U $µl^{-1}$ (EP0404, Thermofisher scientific, USA) and nuclease free water. The thermal cycling conditions used were as follows: Initial denaturation of 94°C for 5 min, followed by 10 cycles of 94°C for 40 sec, 58°C for 40 sec, and 72°C for 1 min; followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; with a final extension step at 72°C for 10 min. The amplified PCR products were stored at 4°C until further analysis. The amplicons size were determined by electrophoresis employing 2.5% agarose gel (Sigma) in 1xTAE buffer. The size of the amplicons were compared to the 50 bp plus DNA molecular weight marker (Thermofisher scientific, USA) and documented in a gel documentation system.

The screening of seafood as well as seafood market/ aquaculture environment of coastal Kerala for MRSA revealed that out of the 233 samples, 13.4% samples carried MRSA. Moreover, it is pertinent to note that 36.5% of the *S. aureus* positive samples were found to harbour MRSA. The prevalence of MRSA in finfish, crustaceans, molluscs and in the environment were 13.8, 9.3, 12 and 15.3% respectively. Out of the 31 positive samples harbouring MRSA, 64.5, 32.2 and 3.3% belonged to Ernakulam, Kottayam and Alappuzha districts respectively. Incidence of *S. aureus* and MRSA in different sample types are depicted in Fig. 1. The results from this study revealed a higher prevalence of MRSA in comparison to that of 5% MRSA reported in ready-to-eat raw fish samples from fish market by Hammad *et al*. (2012) in Japan. Costa *et al.* (2015) reported presence of MRSA in raw fish and processed fish in hospital kitchen at 30 and 15% of the samples respectively.

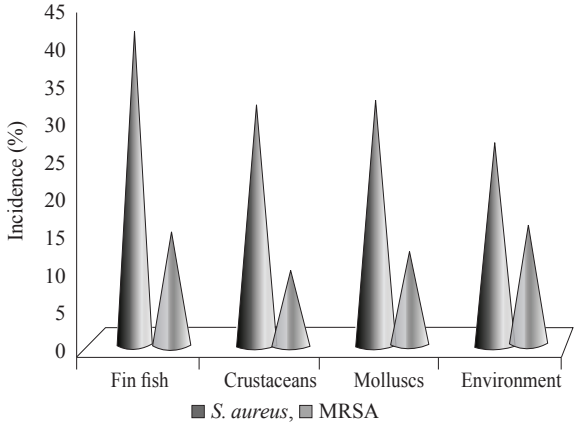

Fig. 1. Distribution of *S. aureus* and MRSA in seafood and fishery environment from south-west coast of India

In the present study, based on the results of antimicrobial susceptibility and multiplex PCR, it was confirmed that the methicillin resistance of the isolates were mediated by *mecA* gene and not by non-*mecA* mediated resistance. All the MRSA strains gave amplicons of 310bp for *mecA*, 750 bp for 16s rDNA gene and 280 bp for *nuc* gene, whereas *S. aureus* produced only 16s rDNA and *nuc* gene specific products (Fig. 2). Zhang *et al*. (2004) were able to differentiate *S. aureus* from MR-CoNS by multiplex PCR.

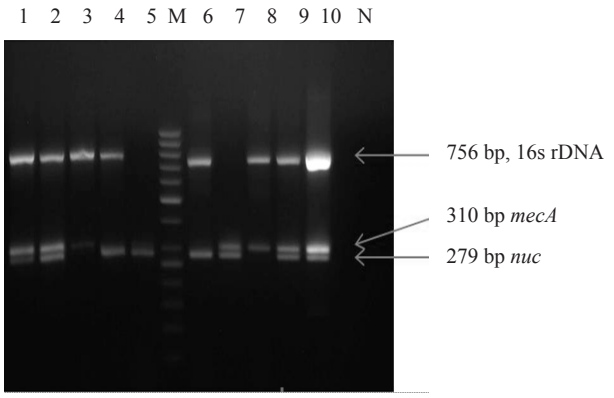

Fig. 2. Multiplex PCR for detection of MRSA
Lane1&2: MRSA strains from samples; Lane 9&10: MRSA positive controls ATCC 43300, ATCC33592;
Lane 3&8: MR-CoNS sample strain; Lane4&6: MSSA strain; Lane 5&7: non staphylococci; Lane M: 50 bp molecular weight marker

Hammad *et al*. (2012) used BP agar with cefoxitin selective screening of MRSA/MR-CoNS; while Costa *et al.* (2015) used two enrichment procedures; primary enrichment in Mueller Hinton broth with 6.5% salt and secondary enrichment in phenol red mannitol salt broth with subsequent plating onto selective chromagar plate for detection of MRSA from raw and processed meat including fish. Atyah *et al.* (2010) reported prevalence of MRSA in cultured tilapia from Malaysia, where mannitol salt agar was used as screening medium. Weese (2010) reported that prevalence data may vary considerably according to methods for isolation, sample types and sample collection schemes employed. It is likely that protocols used for determining the prevalence of MRSA from fish samples may affect the prevalence rate and hence there is a need for harmonised protocol for detection of MRSA from food samples. Weese (2010) and Costa *et al.* (2015) have indicated that there is considerable increase in prevalence of MRSA in food animals and it is imperative that MRSA food poisoning may become more common in future. The study concludes that there is a prevalence of 13.4% methicillin resistant *S. aureus* in samples of fish, fishery products as well as market/ aquaculture environments. Hence there is a need to alert the fish farmers and fish handlers to adopt strict hygienic measures during harvest, handling and storage of fish. Further studies are required to trace the source of contamination by MRSA in seafood and fishery environment.

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