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Short communication

Tracing contamination of Methicillin-resistant *Staphylococcus aureus* (MRSA) into seafood marketing chain by staphylococcal protein A typing

Vaiyapuri Murugadas*, Toms C. Joseph, Kuttanapilly V. Lalitha

MFB Division, ICAR - Central Institute of Fisheries Technology, CIFT Junction, Willingdon Island, Matsyapuri P.O., Cochin 682 029, Kerala, India

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is not a resident flora of fish. Its presence is endorsed to post-harvest contamination viz., handlers, handling equipment and environment. Earlier investigation revealed the presence of MRSA in seafood sold in retail fish markets in Kerala, India. Further studies were conducted to understand and identify the source of contamination into seafood sold in the retail markets by a pilot study. Seventeen samples which includes seafood and fishery environment samples from a landing centre and a retail fish market were collected to identify the source of contamination of MRSA. The whole experiment was repeated with same sampling plan for validation of the procedure, a week later from the same landing centre to the point of sale at fish market. MRSA was isolated from 35.2% to 23.5% of samples during first and second visits respectively. *spa* typing of the MRSA isolates revealed that MRSA from the landing centre (t311 and t15669) were carried to the retail fish market. Ice and water were the probable source for contamination during handling at the landing centre. This is first study to trace the source of contamination of MRSA in seafood and fishery environment. It is imperative that *spa* typing can be implemented for studying the local spread of MRSA clones at specific geographical locations only after establishing its diversity. To better understand the complexity of local spread of MRSA and reproducibility of this experiment, studies has to be conducted in other landing centre and retail markets.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported from hospitals (Jevons, 1961). Over past two decades it has taken various dimensions *viz*., first as hospital acquired MRSA (HA-MRSA) (Barrett, McGehee, & Finland, 1968) in the form of epidemic, pandemic and endemic in certain geographical locations of health care facilities; second as Community acquired (CA-MRSA) infection that occurred outside of the healthcare setup in 1990's (Saravolatz, Markowitz, Arking, Pohlod, & Fisher, 1982) and a dimension as Livestock associated (LA-MRSA) in food producing animal as a possible vehicle of transmission between animals and humans (Voss, Loeffen, Bakker, Klaassen, & Wulf, 2005; de Neeling et al., 2007). Food borne outbreak due to HA-MRSA and CA-MRSA has been reported (Doyle, Hartmann, & Wong, 2011; Jones, Kellum, Porter, Bell, & Schaffner, 2002; Kluytmans et al., 1995). MRSA or *S. aureus* is not a native flora of fish. Its presence is due to post-harvest contamination from handlers, handling equipments and environment (Huss, 1988, p. 132; Simon & Sanjeev, 2007) along the food production chain from capture to landing and processing. During the processing period fish amicable for contact with surfaces of handling equipment, storage environment and washing water within the production environment. Contamination by microorganisms occurs through either water, personnel or inadequate cleaning procedures. Several investigations were performed on food, food producing animals (Kamal, Bayoumi, & Abd El Aal, 2013; Tenhagen, Arth, Bandick, & Fetsch, 2011) and food handlers (Ferreira et al., 2014) on prevalence of MRSA. However, presence of MRSA in fish or seafood were being documented since 2010 (Arfatahery, Davoodabadi, & Abedimohtasab, 2016; Atyah, Zamri-Saad, & Siti-Zahrah, 2010; Rhee & Woo, 2010; Hammad, Watanabe., Fujii, & Shimamoto, 2012; Sergelidis et al., 2014) including India (Kumar, Kasim, Lekshmi, Nayak, & Kumar, 2016; Murugadas, Joseph, Reshmi, & Lalitha, 2016; Visnuvinayagam, Joseph,

* Corresponding author.
E-mail address: drmurugadas@gmail.com (V. Murugadas).

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Murugadas, Chakrabarti, & Lalitha, 2015). MRSA clones, either community or hospital associated, were found in aquatic environments such as coastal, marine and freshwater recreational beaches (Levin-Edens et al., 2012; Roberts, Soge, & No, 2013; Soge, Meschke, No, & Roberts, 2009) and hence has the potential to enter the seafood chain.

Even though MRSA has not been considered as a foodborne pathogen (Wendlandt, Schwarz, & Silley, 2013), the occupational hazard associated with their presence in seafood generally cannot be neglected. This necessitates rapid detection and tracing the source of contamination (Weiser et al., 2016). There is an urgent need for identifying the contamination points in seafood marketing chain; from aquaculture farm/fishes to table and elucidating the transient/persistent microbial contaminants in the food (Reskova, Korenova, & Kuchta, 2013). Foley, Lynne, and Nayak (2009) demonstrated the availability of molecular tools for microbial source tracking and epidemiological investigations of food borne pathogens and categorized these tools into three broad types; based on restriction analysis (MLEE, PFGE, Plasmid profiling, RFLP, Ribotyping), amplification of genetic targets (AFLP, RAPD, Rep-PCR, VNTR, MLVA) and DNA sequence polymorphism at specific loci in the genome (MLST, SNPs). Application of these molecular typing method in food production chain requires high throughput, speed, simplicity and low cost (Sabat et al., 2013) and better discrimination. Concept of tracing the source of contamination has been implemented by various researchers viz., RAPD for Listeriosis (Zhou & Jiao, 2004); rapid biochemical methods for Coliforms in food processing factories (Tominaga, Sekine, & Oyaizu, 2008); Multilocus variable number of tandem repeats (MLVA) as a tool in tracing source of contamination in food processing environment (Reskova et al., 2013; Koreňová, Rešková, Véghová, & Kuchta, 2015) and *spa* typing for *S. aureus* (Ho, Boost, & O'Donoghue, 2015). Roussel et al. (2015) has demonstrated the good epidemiological concordance of the four typing methods viz., *spa* typing, MLVA, PFGE and PCR (Staphylococcal Enterotoxins genes) for the rapid characterization of *S. aureus*. However the gold standard for MRSA typing (PFGE) is not recommended for the reason of non-typeability of Animal-Associated MRSA. Now sequence-based typing methods (MLST and *spa* typing) are commonly used for typing Animal Associated -MRSA in epidemiological studies (Rasschaert et al., 2009). *spa* and MLST are comparatively good discriminating tools with advantages of rapidity, interlaboratory reproducibility and easiness to perform which makes them the first line of typing method till today. MLST is laborious, cost intensive, often associated with the limited number of *spa* types, commonly useful for long term and global epidemiological investigation in contrary to *spa* typing which is useful for short-term, local epidemiological investigation for micro-variation within a geographical region (Monecke et al., 2011; Wendlandt et al., 2013) and hence *spa* typing was used in the local transmission of MRSA clones studies.

Keeping these in view, a null hypothesis was committed; there is no transmission or local spread of MRSA between landing centre and retail fish market, and water or ice are not the possible source for contamination of seafood. To address these researchable points a pilot study was conducted from a landing centre to a retail market in Kerala, India twice to trace the source of contamination.

2. Material and methods

2.1. Study plan

A sampling plan was carried out for tracing the source of contamination (MRSA) into seafood after studying prevalence of MRSA in seafood in retail fish market (Murugadas et al., 2016). The sampling started at the landing centre (Chempu, Murinjampuzha, Kottayam district, Kerala, India) and ended at retail fish market (Polakkandam, Ernakulam district, Kerala, India) on the same day within 2 h, where a small fraction of the seafood landed were transported to this market for sale by vendors. Seventeen samples that included, ten samples from landing centre and seven samples from retail fish markets were collected in sterile sample bags/plastic/glass containers depending on the type of sample. Sampling plan was repeated within a week to validate the procedure with same sample number. Samples collected during this study (Table 1) were brought to the laboratory in insulated chilled box and processed within 3 h of sampling.

2.2. Isolation of MRSA

Ten gram of sample was homogenized and transferred to 90 mL TSB containing 10% NaCl and 1% sodium pyruvate and incubated at 35 °C for 24 ± 2 h (Bennett & Lancette, 2001) followed by streaking on Baird-Parker (with potassium tellurite and egg yolk supplement) agar and BD chromAgar MRSA II (Cat.No.215228, BD Difco, USA) and incubated the plates at 35 °C for 24–48 h. Typical colonies from BPA agar and BD ChromAgar MRSA II were transferred to Mannitol salt agar and Oxacillin Resistance Screening agar base (ORSAB; Cat.No.CM1008, Oxoid, UK) with ORSAB supplement (Cat.No. SR0195, Oxoid, UK) and incubated at 35 °C for 24 ± 2 h.

Isolates were tested for its morphological and biochemical reactions viz., Gram's reaction, catalase, oxidase, glucose and mannitol fermentation and coagulase test. Phenotypic oxacillin resistance was assessed by agar dilution method as per CLSI (2014) in Mueller-Hinton agar (MHA) with 4% NaCl and oxacillin 6 μg/mL and disk diffusion assay with cefoxitin (30 μg) disk. *S. aureus (*ATCC 29213 − MSSA) and *S. aureus* ATCC 43300 (MRSA) were used as negative and positive control respectively.

2.3. Molecular confirmation by mPCR

Molecular confirmation of MRSA was performed by mPCR (Zhang et al., 2004) targeting 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), and *mecA* (a determinant of Methicillin resistance) genes. mPCR assays were performed using crude bacterial DNA extract prepared by boiling lysis method. 3 μl of template was added to a 22 μl PCR mixture containing 50 mM KCl, 20mMTris-HCl (pH 8.4), 2.5mMMgCl2, 200 μM dNTPs Mix (Thermofisher scientific, USA), 0.12 μM each 16S rRNA and *mecA* primers, 0.04 μM each *nuc* primer, and 1.0 U of *Taq* DNA polymerase Recombinant (Thermofisher scientific, USA). Amplification was performed in Veriti Thermal cycler (Thermofisher scientific, USA). Amplification cycle was as follows: an initial denaturation step at 94 °C for 5 min; 10 cycles of 94 °C for 40 s, 58 °C for 40 s, 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.

2.4. spa typing for tracing the contamination into seafood

Staphylococcal protein A typing (*spa* typing) was performed (Koreen et al., 2004; Strommenger et al., 2008) using the primers spa -1113f TAAAGACGATCCTTCGGTGAGC (22bp) and spa-1514r CAGCAGTAGTGCCGTTTGCTT (21bp) in a 50 μl reaction mix with the PCR reaction mix containing 200 μM dNTP's, 10 pmol concentration of each primer, 5 μl of 10× TakaraExtaq buffer (Takara, Japan) 2 mM Mgcl2, 1.U of Takara Extaq Hotstart DNA polymerase and template DNA of 3 μl. The Cycling conditions followed were Initial denaturation at 94 °C for 5min, followed by 35 cycles of Denaturation 94 °C for 45 s, Annealing 60 °C for 45 s, Extension 72 °C for 90 s, followed by a Final extension at 72 °C for 10min. The