Advances in Microbiology, 2016, 6, 399-406
Published Online May 2016 in SciRes. http://www.scirp.org/journal/aim
http://dx.doi.org/10.4236/aim.2016.66039

Scientific Research Publishing

Incidence of Methicillin-Resistant Staphylococci in Fresh Seafood

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Received 6 February 2016; accepted 7 May 2016; published 11 May 2016

Abstract

The occurrence of methicillin-resistant staphylococci was investigated in fresh seafood, seafood products and related samples. Staphylococci were isolated from 13 (68.42%) fresh seafood samples, while 3 (15.78%) samples harbored coagulase-positive *S. aureus.* Resistance to methicillin was observed in 16 isolates of *Staphylococcus* spp., 15 of which were coagulase-negative *S. aureus* (MR-CoNS) and one was a coagulase-positive *S. aureus* (MRSA). The *mecA* gene is detected by PCR in 10 MR-CoNS and one MRSA strain. The *lmrS* gene, which codes for a multidrug efflux pump LmrS, is detected only in coagulase-positive isolates.

Keywords

Seafood, MRSA, CoNS, *mecA*, *Staphylococcus*

1. Introduction

Staphylococcus aureus, an opportunistic bacterial pathogen commonly associated with asymptomatic colonization of skin and the mucosal surfaces of humans and animals, is one of the leading causes of food-borne illnesses in humans [1] [2]. Food poisonings due to *S. aureus* occur when foods containing one or more preformed staphylococcal enterotoxins (SEs) are ingested. *S. aureus* is also responsible for many of the nosocomial infections and community acquired diseases [3]. Emergence of *S. aureus* as a serious pathogen is attributed to its intrinsic virulence and the capacity to adapt to different environmental conditions and also by virtue of its ability to develop or acquire resistance to almost any new antimicrobials [4] [5]. The antibiotic resistance of *S. aureus* has become a major concern following the emergence of MRSA (methicillin-resistant *S. aureus*) and CA-MRSA

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How to cite this paper: Kumar, L.R.G., Kasim, A.K., Lekshmi, M., Nayak, B.B. and Kumar, S. (2016) Incidence of Methicillin-Resistant Staphylococci in Fresh Seafood. *Advances in Microbiology*, **6**, 399-406.
http://dx.doi.org/10.4236/aim.2016.66039

(community-acquired MRSA) [6]. In addition to MRSA, methicillin-resistant coagulase-negative staphylococci (MR-CoNS) are increasingly being recognized as causative agents of nosocomial infections [7]. MRSA have been found in farms and food animals [8], meat [9] [10], milk [11] and poultry [12]. Livestock workers and meat handlers are at particular risk of being colonized by livestock-associated MRSA (LA-MRSA) [13]. Certain lineages of MRSA, such as the type (ST) 398, spa type t108, are capable of human to human transmission or animal to human transmission [14]. Freshly caught seafood is free from *S. aureus* [15], hence their presence on fish is a clear indication of secondary contamination during transport and handling [16]. MRSA is introduced into foods by food handlers who are carriers of this bacterium or contamination by other foods which harbor MRSA. Past studies have reported the occurrence of antibiotic-resistant *S. aureus* in fresh seafood [17], ready-to-eat fish [18], seafood products [19] and also in cultured fish [20]. In this study, we sought to determine the prevalence of methicillin-resistant staphylococci in fresh seafood, fish products, seafood processing equipment and the sea salt used in fish fermentation. The results of this study show that MR-CoNS are more dominant than the MRSA in fresh seafood. This is the first report on the incidence of methicillin-resistant staphylococci in fresh seafood in India.

2. Materials and Methods

2.1. Sampling

Thirty-five samples were processed for the isolation of *Staphylococcus* spp. which included seafood samples from retail markets and landing centers in North Mumbai, fish products, salt, seawater and surface swabs. Sterile swabs were used to collect duplicate swab samples from the surfaces of processing equipment such as the silent cutter, mixer, extruder as well as the pre-processing table in the fish processing unit of the institute where this study was conducted. Moistened swabs were rolled multiple times on the surfaces and rinsed in sterile saline followed by spread plating of 0.4, 0.3 and 0.3 ml of suspension on Baird Parker agar.

2.2. Isolation and Identification of *S. aureus*

Twenty-five grams of the sample (fresh fish or fish products) was aseptically weighed and mixed with 225 mL tryptone water and homogenized for 60 seconds in a stomacher (Seward Stomacher 80, Lab system, London, UK). The homogenate was serially diluted with sterile saline (1:10 dilution). From each dilution, aliquots of 0.4, 0.3 and 0.3 ml each were spread plated on Baird-Parker agar (BPA) supplemented with 3.5% egg yolk-tellurite emulsion [21]. Colonies typical of *Staphylococcus* spp. picked from BPA plates were Gram stained and identified by biochemical tests, which included catalase test, glucose and mannitol fermentation tests and sensitivity to novobiocin. The coagulase production was determined by coagulase test using rabbit serum (Hi-Media, Mumbai, India).

2.3. Antibiotic Resistance Tests

The methicillin-resistance phenotype of staphylococci was determined by standard disc diffusion method on Mueller-Hinton agar (Hi-Media, Mumbai, India) using oxacillin (1 µg) andcefoxitin (30 µg). The plates were incubated at 35˚C for 24 h. A total of 199 isolates, comprising of 4 coagulase-positive *S. aureus* and 195 CoNS, were included in the test. The results of antibiotic susceptibility test were interpreted as per guidelines of CLSI for *S. aureus* and CoNS [22].

2.4. Oligonucleotide Primers and PCR

Oligonucleotide primers specific for *Staphylococcus* genus-specific 16S *rDNA* gene, *S. aureus* species specific *femA* gene (encoding a factor responsible for methicillin resistance) and the *nucA* gene (encoding a thermonuclease), methicillin-resistance gene *mecA* and the lincomycin efflux gene *lmrS* were used to in the PCR assays (**Table 1**). DNA of *Staphylococcus aureus* ATCC BAA-976) was used as the positive control in all PCR reactions. The thermocycling conditions for the amplifications of 16S rDNA, *femA*, *nuc* and *mecA* genes were same as previously described [23]-[25]. For the amplification of *lmrS* gene using the primers designed in this study, the thermocycling conditions consisted of 1 min denaturation at 94˚C, 1 min annealing at 55˚C and 1 min extension at 72˚C. The products of PCR were electrophoresed on 1.6% agarose gel, stained with ethidium bromide and photographed using a gel documentation system (Bio-Rad, Hercules, USA)

Table 1. Oligonucleotide primers used.

Primer	Nucleotide sequence (5'-3')	Product size (bp)	Reference
16S rDNA	gcaagcgttatccggattt cttaatgatggcaactaagc	599	25
femA	cgatccatatttaccatatca atcacgctcttcgtttagtt	454	25
nuc	gcgattgatggtgatacggtt agccaagccttgacgaactaaagc	270	23
mecA	actgctatccaccctcaaac ctggtgaagttgtaatctgg	163	24
lmrS	aaatggtactcgccaactcg tggcgtcatgatacctctga	241	This study

3. Results

Different sample types analyzed in this study yielded *Staphylococcus* spp. (**Table 2**). Among seafood samples, staphylococci were isolated from 10 of 14 fish samples and 3 of 5 shellfish samples, for an overall prevalence rate of 68.42%. Of various seafood products, 3 of the 10 products yielded staphylococci. These products included breaded and battered tuna, fish sausage and fish pickle. A total of 199 isolates were confirmed to be *Staphylococcus* spp. by biochemical tests as well as by the genus-specific 16S rDNA PCR. When different samples were compared, the highest incidence of *Staphylococcus* spp. was found in fish samples followed by fish products and shellfish (**Table 2**). Of the 4 swab samples collected from the fish processing unit, 3 samples yielded *Staphylococcus* spp. The coagulase-positive *S. aureus* were found in 4 samples of which 3 were fish samples and one was a sample of sea salt used in fish fermentation. These isolates were identified by *S. aureus*-specific PCRs amplifying *nucA* and *femA* genes. The coagulase-positive *S. aureus* were isolated from fresh *Tenulosailisha*, *Coilia dussumieri* and dried *Harpadon nehereus* (Bombay duck).

Both coagulase-positive *S. aureus* and coagulase-negative *Staphylococcus* spp. were tested for methicillin resistance phenotype. The different sample types that yielded methicillin-resistant staphylococci are shown in **Table 3**. Of the 199 isolates of *Staphylococcus* spp. tested, 16 isolates from 9 samples were resistant to oxacillin/cefoxitin, of which 1 was a coagulase-positive *S. aureus*. This particular *S. aureus* was isolated from the salt. The remaining 3 *S. aureus* isolates were sensitive to methicillin. The *mecA* gene, which encodes penicillin-binding protein PBP2a, was detected by PCR in 10 methicillin-resistant coagulase-negative staphylococci (MR-CoNS) and one MRSA isolate. Five MR-CoNS isolates were negative for *mecA* gene. The *lmrS* gene was detectable in 4 coagulase-positive *S. aureus* isolates, one of which was a MRSA. None of the MR-CoNS carried the *lmrS* gene (**Figure 1**).

4. Discussion

Several factors such as poor hygiene and sanitation during seafood handling and transportation, cross-contamination during storage and contamination by workers who are asymptomatic carriers of coagulase-positive *S. aureus* contribute to the introduction of *S. aureus* into the seafood. Many studies have shown that *S. aureus* could be present in fresh seafood, ready-to-cook and ready-to-eat seafood products, seafood processing environments and the hands of seafood handlers [26]-[32]. In our study, a total of 35 random samples were analyzed for the presence of *Staphylococcus* spp. and the bacterium was isolated from 20 samples. Coagulase-positive *S. aureus* were found in 4 samples, of which 3 were fresh fish samples and one was a sample of salt used in fish fermentation (**Table 2**). The occurrence of coagulase-positive *S. aureus* in fresh seafood is 15.78% (3 of 19 samples). Different studies have recorded varying rates of *S. aureus* isolation from seafood. Normanno *et al*. [33] reported a low isolation rate of 2.3% from fish products, whereas a higher incidence of 20% was reported in fresh seafood harvested in the southern region of Brazil [27]. A recent study by Zarei *et al*. [34] detected *S. aureus* in 5% of the raw/fresh samples of fish and shrimp, 17.5% of the frozen, and 12.3% of the RTE samples marketed in Iran. A relatively high incidence of *S. aureus* has also been reported from Spain in which *S. aureus* was found in 43% of fresh fish and 30% of frozen products [28]. A pervious study from India has reported that 17% of the fishery products and 62% of the samples from the factory workers were positive for enterotoxigenic *S. aureus* [32]. However, this high prevalence rate was found in frozen peeled shrimps and cuttlefish which were subjected

Table 2. Occurrence of *Staphylococcus* spp. in different sample types.

Sample type	No. of samples analyzed	No. positive for staphylococci	No. positive for *S. aureus*
Fish	14	10	3
Shellfish	5	3	-
Fishery products	10	3	-
Fish processing environment	4	3	-
Salt	1	1	1
Seawater	1	0	-
Total	35	20	4

Table 3. Distribution of methicillin-resistant staphylococci in samples analyzed in this study. The methicillin resistance was determined using oxacillin (1 µg) and cefoxitin (30 µg) disks.

Samples	No. of isolates tested	No. resistant to methicillin
Coilia dussumieri	7	0
Tenulosa ilisha	4	0
Epinephelus diacanthus	7	0
Nemipterus japonicus	3	0
Muraenoscox cinereus	2	0
Harpadon nehereus	2	0
Pampus argenteus	31	5
Trichiurus lepturus	14	0
Tachysaurus dussumieri	20	1
Dried *Harpadon nehereus* (Bombay duck)	4	0
Metapenaeus dobsoni	4	2
Meretrix meretrix	7	1
Loligo duvacelli	9	0
Fish sausage	15	1
Battered and breaded tuna	24	3
Fermented Indian mackerel (*Rastrelliger kanagurta*)	34	1
Salt	1	1[a]
Swabs from silent cutter	4	1
Swabs from extruder	7	0
Swabs from pre-processing table	1	0
Total	199	16

to extensive handling at various stages of processing leading to higher levels of contamination.

In our study, coagulase-positive *S. aureus* was isolated from a sample of salt used in fish fermentation and preparation of fish products in our institutional facility. *S. aureus* is a common contaminant of salt since it can tolerate low water activity. When such salt is used in the preparation of salted fish or other fish products, *S. aureus* is introduced into the products [35]. Isolation of *S. arlettae* in large numbers from salted cod has been reported and this species was found to be extremely halotolerant, being able to grow from 0.06 M - 4.5 M NaCl [36]. High prevalence of *S. aureus* has been reported in salted fish, smoked and salted fish, and salted and cold smoked fish [19] [37] [38]. *S. aureus* is a known halotolerant organism, being able to grow at salt concentrations

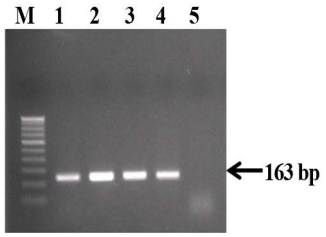

Figure 1. Detection of *lmrS* gene in seafood isolates of coagulase-positive *S. aureus*. Lane M, Gene Ruler 1 kb DNA ladder (Fermentas); Lane 1, Positive control *S. aureus* BAA-976; Lane 2, isolate from salt; Lanes 3 & 4, isolates from fish; Lane 5, negative control.

of 7% - 10%, with some strains being able to withstand a NaCl concentration as high as 20% [39]. In salted sardines, *S. aureus* was reportedly able to survive for up to 90 days [40]. However, a literature search did not yield any information on the isolation of *S. aureus* directly from the salt. Nevertheless, the isolation of toxigenic *S. aureus* from an additive used in the preparation of fish products assumes significance. It is important to ensure that good quality raw materials and additives are used in the preparation of fishery products to prevent the spread of toxigenic *S. aureus* in general, and methicillin-resistant *S. aureus* in particular, since the isolate from salt in this study was found to be a MRSA.

A total of 199 isolates were identified as *Staphylococcus* spp. by 16S *rDNA* PCR. Of these, 4 coagulase-positive isolates were confirmed to be *S. aureus* by thermonuclease gene (*nucA*)-specific PCR. We also used a previously described PCR method amplifying *femA* gene to discriminate coagulase-positive *S. aureus* from CoNS [41]. The product of *femA* gene is essential for the expression of methicillin resistance in *S. aureus* and this gene has been reported to be a marker for *S. aureus*. All coagulase-negative staphylococci (CoNS) of this study were negative for *femA*.

Both MRSA and MR-CoNS are recognized worldwide as zoonotic agents capable of causing serious human infections and their presence in foods is a serious human health concern [42]-[44]. The focus of this study was also to understand the prevalence of MRSA and MR-CoNS in seafood. Based on cefoxitin and oxacillin resistance, a total of 16 isolates were found to be resistant to methicillin, of which one was a coagulase-positive *S. aureus* (**Table 3**). A PCR assay for *mecA* gene amplified the gene in 11 out of 16 isolates. Five MR-CoNS isolates were negative by *mecA* PCR. It is possible that the *mecA*-negative MR-CoNS may have a different *mecA* gene or a different mechanism of methicillin resistance altogether [45]-[47]. Some MR-CoNS isolated from sashimi were reported to be *mecA* negative [18]. Further, only 27.9% of the methicillin-resistant staphylococci were found to carry *mecA* gene by PCR [48]. Nevertheless, the presence of MRSA in seafood and seafood products is increasingly being reported creating health concern. MRSA have been isolated from fresh fish [49], cage cultured Tilapia [20] and the Japanese retail ready-to-eat raw fish (sashimi) [18] and other fishery products [17].

Efflux pumps are employed by bacteria to expel antimicrobial compounds including antibiotics out of the cell and protect themselves from their lethal effects [50] [51]. Such multidrug transporters can contribute significantly to bacterial multiple drug resistance (MDR), thus reducing the efficacy of chemotherapy. The genome of *S. aureus* contains more than 20 efflux pumps, majority of which are of the Major Facilitator Superfamily (MFS) type. MFS includes highly related secondary active and passive solute transporters, widely recognized as responsible for intrinsic and acquired antibiotic resistance in bacteria. Recently, Floyd *et al*. [52] described a multidrug efflux pump LmrS in an isolate of MRSA. LmrS can confer high antibiotic resistance to several antibiotics, the most prominent of them being lincomycin, fusidic acid, linezolid and erythromycin. However, not much is known about the distribution of this gene in *Staphylococcus* spp. We therefore wanted to determine whether *lmrS* is present in all *S. aureus* and CoNS and if it could be used as a genetic marker to detect *S. aureus* by PCR. The primers designed in this study detected *lmrS* in all 4 isolates of *S. aureus*, one of which was a MRSA (**Figure 1**). None of the CoNS was positive for the *lmrS* gene. These results are interesting and suggest that *lmrS* is limited to *S. aureus*, but is not a marker for methicillin resistance. Further studies are needed to understand if the isolates harboring *lmrS* are clonal and also if *lmrS* has any role in the physiology of survival of *S. aureus* in seafood, biofilm formation and even resistance to biocides used in fish processing plants.

Acknowledgements

The authors thank Director, CIFE, Mumbai for helpful suggestions.

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