The Pharma Innovation Journal 2022; 11(12): 6009-6013

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(12): 6009-6013

www.thepharmajournal.com
Received: 04-10-2022
Accepted: 10-11-2022

Anjusha KM
PG Scholar, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala, India

Prejit
Associate Professor and Head, Officer-in-Charge, COHEART, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala, India

Hariharan R
Ph.D. Scholar, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala, India

Jess Vergis
Assistant Professor, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala, India

Asha K
Assistant Professor, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala, India

Vinod VK
Assistant Professor, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala, India

Prasanna KS
Assistant Professor and Head (In-Charge), Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy, Kerala Veterinary, and Animal Sciences University, Pookode, Wayanad, Kerala, India

Corresponding Author:
Anjusha KM
PG Scholar, Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala, India

Assessment of post-harvest contamination of seafood with methicillin-resistant *Staphylococcus aureus* in retail markets of Wayanad district, Kerala, India

Anjusha KM, Prejit, Hariharan R, Jess Vergis, Asha K, Vinod VK and Prasanna KS

Abstract
The production and supply chains both are susceptible to post-harvest microbial contamination of seafood. This would result in the propagation of drug-resistant pathogens of public health significance. The present study aims to determine the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) from different retail fish markets in the Wayanad district. A total of 180 fish samples, comprising finfishes (60), crustaceans (60), molluscs (60), and 36 market environmental (handwash, surface swab, and ice) samples, were analyzed for *S. aureus* and MRSA by conventional culture-based and molecular methods. The characterization of isolates for Methicillin resistance by phenotypic disc diffusion method showed more resistance towards oxacillin (11.64%), followed by cefoxitin (5.47%). Molecular confirmation using the *nuc* gene showed the occurrence of *S. aureus* in 90% of fin fishes, 41.6% of crustaceans, 71.6% of molluscs, and 66.67% of environmental samples, respectively. The occurrence of *mecA* gene was found to be 4.09% in seafood and 12.5% in environmental samples.

Keywords: Marine fishes, *mecA*, MRSA, *nuc*, retail outlets

1. Introduction
Post-harvest microbial contamination of seafood can occur at any point during the production and supply chain. The occurrence of pathogens in seafood coupled with the transmission of antimicrobial-resistant genes into the human food chain is the most pressing public health issue faced by the food industry. Food and Agriculture Organization reported that fish production has increased significantly over the past 60 years, reaching over 179 million tonnes in 2018 and holding a market value of US $401 billion (FAO, 2020) [11]. Approximately, 845 million people are believed to be nutritionally dependent on seafood (Béné *et al*., 2015) [6] and currently, it accounts for up to 20% of animal protein, and it often contains a rich source of vitamins, minerals, and omega-3 fatty acids which are essential for human health (Golden *et al*., 2016) [12]. By 2050, the global human population is projected to reach 10 billion (Springmann *et al*., 2018) [22], influencing socioeconomic demographics to alter and nutritional trends to shift toward more resource-intensive foods (Tilman and Clark 2014; Willett *et al*., 2019) [23, 28], thus seafood will fetch a better market.

In general, fish and seafood get contaminated due to adverse environmental conditions such as contaminated water, seepage of sewage in harvesting area, and from post-harvest contamination through workers, utensils, equipment, and unhygienic handling resulting in presence of a huge count of pathogenic bacteria (Roberts, 2003; Ali, 2014) [18,1]. For decades, farm to fork concept has been concerned about food safety issues arising from a foodborne pathogen such as *S. aureus.* It is the most invasive species, responsible for a wide range of infections, some of which are fatal (Chambers and DeLeo, 2009; Weese, 2010) [9, 29]. Around 30 different virulence or putative factors, such as exfoliative, epidermolytic, and membrane-damaging toxins, were also produced by the organism (Todar, 2017) [24]. MRSA is referred to as 'strains resistant to penicillinase-resistant β-lactams' (methicillin and oxacillin). The resistance is mediated by the *mecA* gene, which encodes for an alternative penicillin-binding protein (PBP2) with poor binding affinity to all β-lactam antibiotics (Chambers and DeLeo, 2009) [9]. The occurrence of *S. aureus* in relation to MRSA in fish and fishery products is least documented and limited reports are available for post-harvest contamination in fishes. Since seafood is handled by various people in the supply chain, the possibility of the occurrence of MRSA in seafood is relatively high.

Under this context, the present study was designed to detect MRSA in seafood and surrounding environmental samples collected from retail fish outlets of Wayanad district of Kerala.

2. Materials and Methods

2.1 Sample collection and isolation

A total of 216 samples comprising finfishes (n=60), crustaceans (n=60), molluscs (n=60), and the retail market's environmental samples (n=36) were collected from June 2021 to June 2022 from fish markets of Wayanad district. The samples were collected in sterile 10% peptone water (HiMedia Laboratories Pvt. Ltd., Mumbai) and transported to the laboratory under aseptic chilled condition and processed as per the standard methodology described by Barrow and Feltham (2003) and Agarwal *et al.* (2003) [5,3] for *S. aureus* isolation. Each sample weighing 25 grams was homogenized and transferred to 5 ml Tryptic soy broth (TSB) incubated overnight at 37º C. The enriched sample (0.50 ml each) was subjected to spread plating onto pre-set Baird-Parker agar base (BPA) supplemented with egg yolk potassium tellurite enrichment and incubated at 37º C for 36 to 48 h. The characteristic jet-black colonies surrounded by narrow halo zone from BPA plates were considered presumptive for *S. aureus*. These suspected colonies were subjected to biochemical tests for confirmation (Gram staining, catalase, coagulase, indole production, methyl red, Voges Proskauer, and citrate utilization tests).

2.2 Antibiotic susceptibility testing

Standard disc diffusion assays were performed using the oxacillin (1 g) and cefoxitin (30 g) discs (HiMedia) on the culture of *S. aureus* recovered from the samples. Overnight-grown cultures in TSB were adjusted to 0.50 McFarland Units (ca. 1.50 x 10^8 CFU/ml) before plating onto Mueller-Hinton agar (BD Difco, USA) and incubated for 16 to 24 hours at 35° C. The results of the susceptibility test were analyzed using the CLSI (2019) [8] guidelines.

2.3 DNA Isolation and PCR conditions

DNA was isolated using the phenol-chloroform method. The primers used in the present study are listed in Table 1. The PCR reaction was performed using TAKARA Ready-mix™ PCR Reaction Mix.

Table 1: Details of oligonucleotides used in the study

Target Organism	Target gene	Primer sequence	Amplicons (bp)	Reference
S. aureus	*nuc*	F:5-'GCGATTGATGGGTGATACGGTT-3' R: 5'AGCCAAGCCTTAGACGAACTAAAGC3'	267	Brakstad *et al.* (1992) [7]
MRSA	*mecA*	F:5'AAAATCGATGGTAAAGGTTGGC-3' R:5'-GTTCTGCAGTACCGGAATTTGC 3'	533	Vannuffel *et al.* (1995) [27]

The isolates were subjected to the PCR amplification of the *nuc* gene for *S. aureus*. The following PCR conditions were followed: initial denaturation of 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 50 sec (denaturation), 57 ℃ for 60 sec (annealing), and 72 ℃ for 1 min (extension); with a final extension step at 72 ℃ for 5 min. For the genotypic detection *mecA* gene, the PCR conditions were as: initial denaturation of 94 ℃ for 5 min, followed by 35 cycles of 94℃ for 30 sec (denaturation), 57 ℃ for 30 sec (annealing), and 72℃ for 30 sec (extension); with a final extension step at 72℃ for 5 min. The amplified PCR products were stored at 4℃ until further analysis. The amplicon size was determined by electrophoresis employing 1.50% agarose gel (Sigma) in 1x TAE buffer. The size of the amplicons was compared to the 1000 bp plus DNA molecular weight marker (ThermoFisher Scientific, USA) and documented in a gel documentation system (BioRad, USA).

Fig 1: Jet black colonies with a halo zone around on BP agar

3. Results and Discussion

In the present study, a total of 216 samples were evaluated for detection of *S. aureus* and MRSA, including finfishes (60), crustaceans (60), molluscs (60), hand wash (12), surface swabs (12), and ice (12) samples from different retail markets of Wayanad district. For the isolation, the samples were enriched in TSB and plated onto BP agar to obtain greyish-black colonies with a halo around them that are presumptively identified as *S. aureus* (Fig. 1) and further confirmed by biochemical tests. This study reported the incidence of MRSA in fish and the retail environmental samples collected from different retail fish markets of the Wayanad district of Kerala, India. The detection of *S. aureus* by selective isolation, antimicrobial resistance profiling, and molecular confirmation of the isolates encoding the *mecA* gene was also studied.

3.1 Occurrence of S. aureus in finfishes, crustaceans, and molluscs

S. aureus was detected in 90% of the samples of finfish collected from different retail markets in the Wayanad district (54 samples). The results obtained in this study coincide with the similar findings of Vishnuvinayagam *et al*. (2015) [27] who reported a prevalence rate of 93%. Our results also showed a higher prevalence than several other studies conducted by Sahu *et al*. (2012) [19] with a prevalence of 14% and Mus *et al*. (2014) [15] reported 3.8%. The findings of a higher occurrence rate in our study may be due to post-harvest handling through the contaminated hands of vendors.

The occurrence of *S. aureus* was found to be 41.66% in 60 samples of crustaceans (25 samples) and it is slightly higher than the result obtained by Murugadas *et al.* (2017) [16] where

the prevalence rate obtained was 36.5%. None of the samples tested was found contaminated by *S. aureus* as per the study conducted by Duarte *et al.* (2021) and Hatha *et al.* (2003) [10,13]. This higher prevalence may be an indication of post-harvest contamination and also due to poor personal hygiene or fish diseases.

The occurrence of *S. aureus* from molluscan samples was found to be 71.66% (43 samples), which was slightly lower than the findings of Hammad *et al.* (2012) [13] where the prevalence rate was 88.88%. In contrast, a still lower prevalence rate (8.33%) was recorded in the study conducted by Mus *et al.* (2014) [15]. The findings of a higher occurrence in this study might be because of inadequate hygienic practices among fish handlers and contact with polluted work surfaces, such as benches, tables, and unwashed knives.

3.2 Occurrence in environmental samples (hand wash, surface swab, and ice)

A total of 12 hand wash samples collected from different retail fish outlets of Wayanad district were screened for *S. aureus* and revealed that it was positive in 75% of the samples (9 samples). The occurrence of *S. aureus* from the surface swabs and ice samples was found to be 75% (9/12 samples) and 50% (6/12 samples), respectively. An overall occurrence was 66.67% in environmental samples which is slightly higher than the results of Albuquerque *et al.* (2007) [2] who reported a 60% prevalence. PCR assay of *nuc* gene depicted in Fig. 2

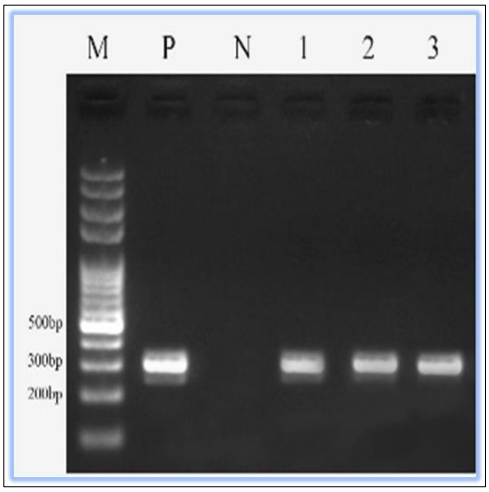

Fig 2: PCR standardisation of *nuc* gene specific for *S. aureus*

Lane 1: 100 bp DNA Ladder Lane 3: Negative control
Lane 2: Positive control Lane 4-6: Positive Samples

3.3 Antimicrobial resistance profiling of MRSA isolates

A total of 54 *S. aureus* isolates were identified from finfish samples and all were subjected to phenotypic MRSA characterization. The results revealed that most of the isolates were resistant to oxacillin (11.11%), followed by cefoxitin (7.4%). Upon phenotypic MRSA characterization of 25 *S. aureus* isolates recovered from crustaceans, most of the isolates showed resistance to oxacillin (24%), followed by cefoxitin (16%). From a total of 43 *S. aureus* isolates obtained from molluscs, most of the isolates showed resistance to oxacillin and none of them revealed resistance to cefoxitin. Nine *S. aureus* isolates were recovered from both hand wash and surface swabs, and six *S. aureus* were identified from ice. All the isolates were found to be sensitive to cefoxitin. In all three isolates, only one was resistant to oxacillin. Among the 146 *S. aureus* isolates obtained from different samples, resistance was mostly observed for oxacillin followed by cefoxitin using a double disc diffusion assay (CLSI, 2019) [8] (Figs. 3 and 4). According to Arfatahery *et al.* (2016) and Sivaraman *et al.* (2021) [4, 21], the resistance towards oxacillin was found to be 23.8 and 100 percent, respectively.

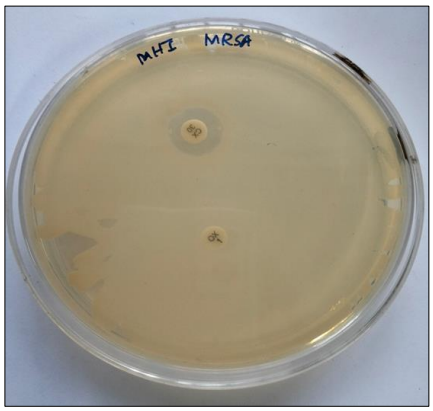

Fig 3: Phenotypic characterisation of MRSA isolates

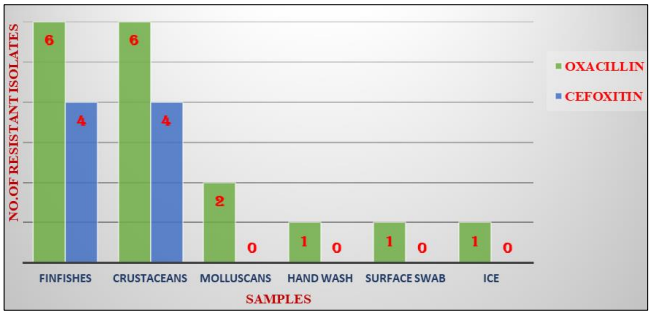

Fig 4: Antibiogram of *S. aureus* isolates

3.4 Genotypic Confirmation of MRSA Isolates

Out of 54 *S. aureus* isolates identified from finfish samples, only one (1.80 percent) isolate was found positive for the *mecA* gene (Fig.5). Among the 25 *S. aureus* isolates recovered from crustaceans, four (16 percent) isolates were positive for the *mecA* gene. However, none of the isolates was positive for the *mecA* gene in molluscs. The *mecA* gene was detected in 11.1%, 11.1%, and 16.7% from handwash, surface swab, and ice samples, respectively. Genotypic identification of *nuc* and *mecA* gene shown in fig 6.

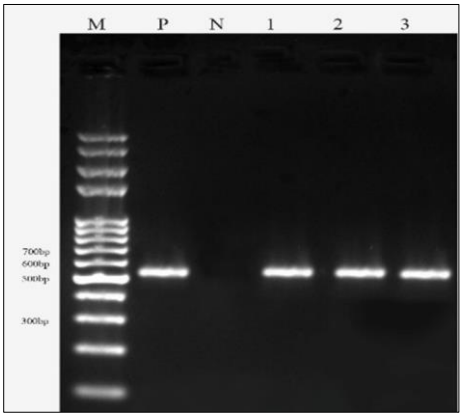

Fig 5: PCR standardisation of *mecA* gene specific for Methicillin Resistance

Lane 1: 100 bp DNA Ladder Lane 3: Negative control
Lane 2: Positive control Lane 4-6: Positive Samples

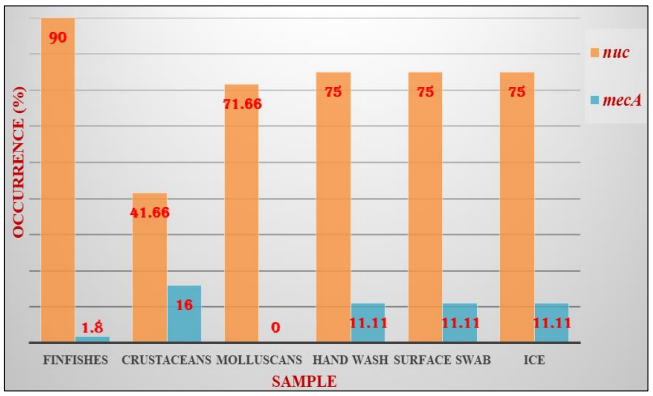

Fig 6: Genotypic identification of *nuc* and *mecA* gene

According to several studies, the prevalence rates of MRSA in fish and fish products ranged between 0.95% to 13.4% in India (Vaiyapuri *et al.*, 2019; Sivaraman *et al.*, 2021; Vishnuvinayagam *et al.*, 2015 and Murugadas *et al.*, 2017) [25, 21, 27, 16]. Similar results are observed in our study, where overall occurrence was 5.47%. Multiple factors, including post-harvest contamination and poor handling practices, are attributed to the occurrence of MRSA in fish products (Murugadas *et al.*, 2017; Sergelidis *et al.*, 2014) [16, 20]. The high prevalence of *S. aureus* in fish samples and its incidence in environmental samples of our study indicate the need for an awareness program for the hygienic handling of fish.

It is presumed that natural water features like rivers serve as collection points for hospital and industrial effluents carrying hazardous pollutants such as antibiotics, radioactive isotopes, disinfectants, and heavy metals, etc. which may lead to several health-associated illnesses (Kaur *et al.*, 2020) [14]. Pathogenic bacteria may tend to develop resistance and spread across the marine ecosystem when pharmaceutical waste enters these rivers, which may be the possible reason for the emergence of antimicrobial genes in seafood (Sivaraman *et al.*, 2021) [21]. In this regard, the majority of hospitals in Kerala were near water bodies raising concerns about the possibility that it could be a source for the emergence of drug resistance to infectious pathogens. The present study revealed the importance to educate and alert fish handlers to implement strict hygiene guidelines during the harvest, handling, and transportation of fish. To determine the source of MRSA infection in seafood and fishing environments, further studies are required.

Acknowledgments
The authors would like to thank the Dean, College of Veterinary and Animal Sciences, Pookode, for providing all necessary facilities and funding to carry out this research work.

References

1. Ali HH. Isolation and identification of Staphylococcus bacteria from fish of fresh water and its antibiotics sensitivity in mosul city. Basrah J Vet. Res. 2014;1(1):33-42.
2. Albuquerque WF, Macrae A, Sousa OV, Vieira GHF, Vieira RHSF. Multiple drug resistant *Staphylococcus aureus* strains isolated from a fish market and from fish handlers. Brazilian J Microbiol. 2007;38:131-134.
3. Agarwal A, Roy I, Gupta MK, Kumar M, Jain A. Prevalence of extended spectrum β-lactamase-producing Gram-negative bacteria in septicaemic neonates in a tertiary care hospital. J Med. Microbiol. 2003;52(5):421-425.
4. Arfatahery N, Davoodabadi A, Abedimohtasab T. Characterization of toxin genes and antimicrobial susceptibility of Staphylococcus aureus isolates in fishery products in Iran. Sci. Rep. 2016;6(1):1-7.
5. Barrow GI, Feltham RKA. Cowan and Steel's Manual for Identif - ication of Medical Bacteria. (3rd Ed) Cambridge press; c2003. p. 331.
6. Béné C, Barange M, Subasinghe R, Pinstrup-Andersen P, Merino G, Hemre GI, *et al*. Feeding 9 billion by 2050–putting fish back on the menu. Food Secur. 2015;7(2):261–274.
7. Brakstad OG, Aasbakk K, Maeland JA. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J Clin. Microbiol. 1992;30:1654-1660.
8. Clinical and Laboratory Standards Institute (CLSI). (2019). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. M100-S24. CLSI, Wayne, PA, USA.
9. Chambers HF, DeLeo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nature Rev. Microbiol. 2009;7(9):629-641.
10. Duarte DAM, Ribeiro AR, Vasconcelos AMM, Silva JVD, de Andrade PLA, Santana AAP. Ocorrência de *Salmonella* spp. e *Staphylococcus* coagulase positiva em pescado no nordeste, Brasil. *Arquivos do Instituto Biológico*. 2021;77:711-713.
11. Food and Agriculture Organization (FAO). The state of world fisheries and aquaculture. 2020. Retrieved from http://www.fao.org/state-of-fisheries-aquaculture.
12. Golden CD, Allison EH, Cheung WW, Dey MM, Halpern BS, McCauley DJ, *et al*. Nutrition: Fall in fish catch threatens human health. Nature. 2016;534(7607):317-320.
13. Hammad AM, Watanabe W, Fujii T, Shimamoto T. Occurrence and characteristics of methicillin-resistant and-susceptible *Staphylococcus aureus* and methicillin-resistant coagulase-negative *staphylococci* from Japanese retail ready-to-eat raw fish. Int. J Food. Microbial. 2012;156(3):286-289.
14. Hatha AM, Maqbool TK, Kumar SS. Microbial quality of shrimp products of export trade produced from aquacultured shrimp. Int. J Food. Microbial. 2003;82(3):213-221.
15. Kaur R, Yadav B, Tyagi RD. Microbiology of hospital wastewater. In Current developments in Biotechnology and bioengineering. Elsevier; c2020. p. 103-148. https://doi.org/10.1016/B978-0-12-819722-6.00004-3.
16. Mus TE, Cetinkaya F, Celik U. Occurrence of *Vibrio*, *Salmonella* and *Staphylococcus aureus* in retail fresh fish, mussel and shrimp. Acta Veterinaria Brno. 2014;83(2):75-78.
17. Murugadas V, Joseph TC, Lalitha KV. Tracing contamination of Methicillin-resistant *Staphylococcus aureus* (MRSA) into seafood marketing chain by staphylococcal protein A typing. Food Control. 2017;78:43-47.
18. Onmaz NE, Abay S, Karadal F, Hizlisoy H, Telli N, Al S. Occurence and antimicrobial resistance of *Staphylococcus aureus* and *Salmonella* spp. in retail fish samples in

Turkey. Mar. Pollut. Bull. 2015;90(1-2):242-246.
19. Roberts RJ. Nutritional pathology. In *Fish nutrition* (453-504). Academic press; c2003.
20. Sahu S, Jana AK, Nath S, Dora KC, Chowdhury S. Management strategies for minimising the incidence of pathogenic bacteria in seafoods at Kolkata fish market. Mgmt. 2012;1(1).
21. Sergelidis D, Abrahim A, Papadopoulos T, Soultos N, Martziou E, Koulourida V, *et al*. Isolation of methicillin-resistant S taphylococcus spp. from ready-to-eat fish products. Letters Appl. Microbiol. 2014;59(5):500-506.
22. Sivaraman GK, Muneeb KH, Sudha S, Shome B, Cole J, Holmes M. Prevalence of virulent and biofilm forming ST88-IV-t2526 methicillin-resistant *Staphylococcus aureus* clones circulating in local retail fish markets in Assam, India. Food Control. 2021;127:108098.
23. Springmann M, Clark M, Mason-D'Croz D, Wiebe K, Bodirsky BL, Lassaletta L, *et al*. Options for keeping the food system within environmental limits. Nature. 2018;562(7728):519-525.
24. Tilman D, Clark M. Global diets link environmental sustainability and human health. Nature. 2014;515(7528):518-522.
25. Todar KG. ed. Todar's online textbook of bacteriology. Kenneth Todar University of Wisconsin-Madison Department of Bacteriology; c2017.
26. Vaiyapuri M, Joseph TC, Rao BM, Lalitha KV, Prasad MM. Methicillin-Resistant Staphylococcus aureus in Seafood: prevalence, laboratory detection, clonal nature, and control in seafood chain. J Food Sci. 2019;84(12):3341-3351.
27. Vannuffel P, Gigi J, Ezzedine H, Vandercam B, Delmee M, Wauters G, *et al*. Specific detection of methicillin resistant Staphylococcus species by multiplex PCR. J Clin. Microbiol. 1995;33:2864-2867.
28. Visnuvinayagam S, Joseph TC, Murugadas V, Chakrabarti R, Lalitha KV. Status on methicillin resistant and multiple drug resistant *Staphylococcus aureus* in fishes of Cochin and Mumbai coast, India. J Environ. Biol. 2015;36(3):571.
29. Willett W, Rockström J, Loken B, Springmann M, Lang T, Vermeulen S, *et al*. Food in the Anthropocene: the EAT–Lancet Commission on healthy diets from sustainable food systems. The Lancet. 2019;393(10170):447-492.
30. Weese JS. Methicillin-resistant *Staphylococcus aureus* in animals. ILAR J. 2010;51(3):233-244.