

SURVIVAL DYNAMICS OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN FISH AND FISHERY PRODUCTS

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by

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DECLARATION

I hereby declare that the dissertation entitled “**SURVIVAL DYNAMICS OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN FISH AND FISHERY PRODUCTS**” is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship fellowship or any other similar title.

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Dated: 30th June, 2018

CERTIFICATE

Certified that the dissertation entitled “**SURVIVAL DYNAMICS OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN FISH AND FISHERY PRODUCTS**” is a bonafide record of independent research work carried out by **Ms. Pooja Saklani** during the period of study from August 2017 to June 2018 under our supervision and guidance for the degree of **Master of Fisheries Science (Post Harvest Technology)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ABSTRACT

Foods that are extensively handled during preparation and stored without refrigeration are usually associated with staphylococcal food poisoning. Staphylococcal food poisoning (SFP) is one of the widespread food-borne diseases in the world following the ingestion of staphylococcal enterotoxins (SEs) which are produced by enterotoxigenic strains of coagulase-positive *S. aureus*. The emergence of methicillin-resistant *S. aureus* (MRSA) has raised concerns on the foods becoming vehicles for the transmission of this pathogen. The present study investigated the survival dynamic of methicillin-resistant *S. aureus* (MRSA) in different seafood matrices stored at different temperatures. (MRSA) was inoculated at 10^7 and 10^4 log CFU levels into all sample group. In fresh Bombay duck stored in ice for 18 days, the MRSA count decreased by 2.75 log CFU/g (42%) and by 0.28 log CFU/g (8%) in 10^7 log CFU and 10^4 log CFU inoculated samples, respectively. However, the total plate count (TPC) in ice-stored Bombay duck increased by 3.02 log CFU/g, from 4.34 log CFU/g to 7.32 log CFU/g by day-16 of storage. In dry Bombay duck stored at room temperature, MRSA count declined by 3.27 log CFU/g (60%) in 10^7 CFU inoculated sample and by 0.91 log CFU/g (25%) in 10^4 log CFU inoculated sample. The total bacterial population also showed decline in number with the counts decreasing by 0.92 log CFU/g at the end of 20-day storage period. However, the populations of MRSA in shrimp stored at -20°C were more stable. MRSA count in frozen peeled shrimp showed 1.52 log CFU/g (28%) reduction in 10^7 log CFU inoculated sample and 0.22 log CFU/g (6%) increment in 10^4 log CFU inoculated sample. Total plate count in frozen peeled shrimp showed 0.92 log CFU reduction in the count. Similarly, the counts of MRSA in cooked and frozen-stored shrimp declined by 1.43 log CFU/g (26%) in 10^7 CFU inoculated sample, while the TPC count showed a minor decrease of 0.371 log CFU/g. The results suggest that methicillin-resistant *S. aureus* survives less efficiently in ice-stored fish and in dry Bombay duck when present in relatively large numbers (7 log CFU in this study), but are better able to survive when present in low population densities (4 log CFU). In contrast, MRSA survives better in frozen-stored shrimp in both high and low population densities, suggesting that temperatures in the range of -20°C are less lethal to this

bacterium. Real-time quantitative PCR for *lmrS* gene which codes for LmrS efflux pump showed that the gene expression was not significantly altered at low temperatures of storage and at high salt concentrations in the medium. To conclude, MRSA showed significant survivability in low temperature preserved seafood and future studies may investigate the physiological and genetic bases underlying this ability of MRSA.

सारांश

तैयारी के दौरान व्यापक रूप से संभाले जाने वाले खाद्य पदार्थ और प्रशीतन के बिना संग्रहित आमतौर पर स्टेफिलोकोकल खाद्य विषाक्तता से जुड़े होते हैं। स्टेफिलोकोकल खाद्य विषाक्तता (एसएफपी) स्टेफिलोकोकल एंटरोटॉक्सिन्स (एसईएस) के इंजेक्शन के बाद दुनिया में व्यापक रूप से खाद्य पदार्थों में से एक है, जो कोगुलेज-पॉजिटिव एस ऑरियस के एंटरोटॉक्सिजेनिक उपभेदों द्वारा उत्पादित होती है। मेथिसिलिन-प्रतिरोधी एस। ऑरियस (एमआरएसए) के उद्भव ने इस रोगजनक के transmission के लिए वाहन बनने वाले खाद्य पदार्थों पर चिंताओं को उठाया है। वर्तमान अध्ययन ने विभिन्न तापमान पर संग्रहीत विभिन्न समुद्री भोजन मैट्रिस में मेथिसिलिन-प्रतिरोधी एस। ऑरियस (एमआरएसए) की गतिशील गतिशीलता की जांच की। (एमआरएसए) सभी नमूने समूह में 10^7 और 10^4 लॉग सीएफयू स्तर पर लगाया गया था। 18 दिनों के लिए बर्फ में संग्रहीत ताजा बॉम्बे बतख में, एमआरएसए की संख्या 2.75 लॉग सीएफयू / जी (42%) और 0.28 लॉग सीएफयू / जी (25%) 10^7 लॉग सीएफयू और क्रमशः 10^4 लॉग सीएफयू इनोक्यूलेटेड नमूनों में कमी आई। हालांकि, बर्फ से संग्रहीत बॉम्बे डक में कुल प्लेट गिनती (टीपीसी) 3.02 लॉग सीएफयू / जी, 4.34 लॉग सीएफयू / जी से 7.32 लॉग सीएफयू / जी भंडारण के दिन 16 तक बढ़ी है। कमरे के तापमान पर संग्रहीत सूखे बॉम्बे बतख में, एमआरएसए की गणना 10^7 सीएफयू इनोक्यूलेटेड नमूने में 3.27 लॉग सीएफयू / जी (60%) और 101 लॉग सीएफयू / जी (25%) द्वारा 10^4 लॉग सीएफयू इनोक्यूलेटेड नमूना में गिरावट आई है। 20 दिनों की भंडारण अवधि के अंत में कुल जीवाणु आबादी में 0.92 लॉग सीएफयू / जी की कमी के साथ संख्या में गिरावट आई है। हालांकि, -20°C पर संग्रहीत झींगा में एमआरएसए की आबादी अधिक स्थिर थी। जमे हुए छिद्रित झींगा में एमआरएसए गिनती 10^7 लॉग सीएफयू इनोक्यूलेटेड नमूना में 1.52 लॉग सीएफयू / जी (28%) reduction दिखाया गया है और 10^4 लॉग CFU / g (6%) 10^4 लॉग CFU inoculated नमूना में। जमे हुए छिद्रित झींगा में कुल प्लेट गिनती 0.92 लॉग इन सीएफयू कमी में कमी आई है। इसी प्रकार, पकाया और जमे हुए भंडारित झींगा में

एमआरएसए की संख्या 1.43 लॉग सीएफयू / जी (26%) 10^7 सीएफयू इनोक्यूलेटेड नमूना में गिरावट आई, जबकि टीपीसी गिनती 0.371 लॉग सीएफयू / जी की मामूली कमी देखी गई। नतीजे बताते हैं कि मेथिसिलिन-प्रतिरोधी एस। ऑरियस बर्फ से संग्रहीत मछली और शुष्क बॉम्बे में कम कुशलता से जीवित रहता है जब अपेक्षाकृत लक्ष्यों की संख्या (इस अध्ययन में 7 लॉग सीएफयू) में मौजूद होता है, लेकिन कम जनसंख्या घनत्व में मौजूद होने पर जीवित रहने में बेहतर होता है (4 लॉग सीएफयू)। इसके विपरीत, एमआरएसए उच्च और निम्न जनसंख्या घनत्व दोनों में जमे हुए संग्रहीत झींगा में बेहतर रहता है, यह बताता है कि -20°C की सीमा में तापमान इस बैक्टीरिया के लिए कम घातक है। एलएमआरएस जीन के लिए रीयल-टाइम मात्रात्मक पीसीआर जो एलएमआरएस इंप्लक्स पंप के लिए कोड दिखाते हैं कि जीन अभिव्यक्ति को भंडारण के निम्न तापमान और मध्यम में उच्च नमक सांद्रता में महत्वपूर्ण रूप से परिवर्तित नहीं किया गया था। निष्कर्ष निकालने के लिए, एमआरएसए ने कम तापमान संरक्षित समुद्री भोजन में महत्वपूर्ण जीवितता दिखाई और भविष्य के अध्ययन एमआरएसए की इस क्षमता के तहत शारीरिक और अनुवांशिक आधारों की जांच कर सकते हैं।

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1. INTRODUCTION

Staphylococci are commonly found on the skin of healthy individuals. About 20% of individuals have persistent colonisation of *Staphylococcus aureus*, whereas 30% are intermittent hosts (Khan *et al*, 2015; Wasserman and Taljaard, 2011). *S. aureus* is present in nasopharynx, perineum, or skin of 30% of healthy people, but can cause infections when host resistance is lowered (e.g., damaged skin) (Chambers, 2001). *S. aureus* is an important and versatile human pathogen that can cause various diseases. Most frequently, it causes skin infections and infections of the respiratory tract (González *et al*, 2003; Monecke *et al*, 2011). Skin infections are usually community-acquired. Among nosocomial pathogens, *S. aureus* is the most common and is responsible for high morbidity and mortality. *S. aureus* pneumonia is frequently encountered in hospitalised patients with underlying conditions, such as in patients suffering from immune deficiencies or viral infections (Ragle *et al*, 2010; NCBI medical microbiology 4th edition). However, *S. aureus* also causes a variety of other severe and life-threatening disease such as the endocarditis, arthritis, toxic shock syndrome, scalded skin syndrome or osteomyelitis (Vyletelova *et al*, 2011; Vikto and Richardson, 2013; Fridkin *et al*, 2005). Certain characters of *S. aureus* such as its ability to adapt to varying conditions within and outside the host, and develop resistance to antibiotics make it a successful pathogen. *S. aureus* possesses diverse virulence factors, many of which are encoded on mobile genetic elements (MGEs) such as plasmids or prophages that can be transferred between strains by horizontal gene transfer (HGT) (Humphreys, 2012; Otto, 2014; Taylor and Unakal, 2017). *S. aureus* is considered one of the most common agents responsible for outbreaks of food poisoning. *Staphylococcal* food poisoning (SFP) is one of the widespread food-borne diseases in the world following the ingestion of staphylococcal enterotoxins (SEs) which are produced by enterotoxigenic strains of coagulase-positive staphylococci (CPS), mainly *S. aureus* (Hennekinne, 2012). The food chain is considered a potential route of transmission of bacteria to human (Khambaty *et al*, 1994).

Staphylococci are found in a wide variety of mammals and birds, and the transfer of *S. aureus* to food is mainly through two primary sources: human carriage during food handling, processing and dairy animals in case of mastitis. Human strains are mainly involved in staphylococcal food poisoning.

Prevention of staphylococcal food poisoning is based on hygiene measures to avoid or reduce contamination of food by *S. aureus*. These procedures must include control of raw materials, proper handling, cleaning and disinfection of equipment. However, as these requirements are usually not sufficient, it is necessary to destroy staphylococci through appropriate treatment, thermal or otherwise, to prevent their growth under refrigerated conditions (Genigeorgis, 1989; Khambaty *et al*, 1994; Hennekinne *et al*, 2012).

Introduction of *S. aureus* into foods occurs by food handlers who are carriers of this bacterium or contamination by other foods and unhygienic conditions. Previous studies have reported the occurrence of antibiotic-resistant *S. aureus* in fresh seafood, ready-to-eat fish, seafood products and also in cultured fish (Atyah *et al*, 2010; Gutiérrez *et al*, 2012). Many factors such as poor hygiene conditions and sanitation during seafood handling, processing, and transportation, cross-contamination during storage and contamination by the food handlers who are asymptomatic carriers of coagulase-positive *S. aureus* and contribute to the introduction of *S. aureus* into the seafood (Kumar *et al*, 2016).

The emergence of multidrug-resistant strains of pathogenic bacteria has made it more difficult to eradicate the diseases they cause. The increasing prevalence of antimicrobial-resistant *S. aureus* is a threat to food safety and healthcare systems. Following the introduction of penicillin in the 1940s, the first penicillinase-producing *S. aureus* strains were detected, which led to the development of penicillinase-resistant semi-synthetic penicillins such as oxacillin, methicillin and the first or second generation antibiotics such as cephalosporins. Methicillin-resistant *S. aureus* (MRSA) was reported in the United Kingdom (UK) in 1961 (Cuevas *et al*, 2007). Resistance towards methicillin is due to modified penicillin-binding protein (PBP2' or PBP2a)

encoded by the *mecA* gene (Monecke *et al*, 2011; Santajit and Indrawattana, 2016). *S. aureus* has developed resistance to various types of antimicrobial drugs, such as second- and third-line antibiotics. Only a few antibiotics like vancomycin (a glycopeptide), daptomycin (a lipopeptide), and linezolid (an oxazolidinone), have been approved for the treatment of severe infections caused by MRSA (Kumar *et al*, 2016). Bacteria, after exposure to antimicrobial agents, may develop drug-resistance mechanisms or in some cases, may be intrinsically resistant to certain antibiotics. These resistance determinants reduce the antimicrobial activities of the drugs. There are four primary molecular mechanisms of bacterial drug resistance (Wright, 2005; Kumar and Varela, 2012).

- i. The enzymatic degradation of the antimicrobial agent
- ii. Alteration of cellular drug target proteins
- iii. Reduced drug permeability
- iv. Efflux pump systems

Efflux pumps are the transport membrane proteins involved in the extrusion of noxious molecules such as an antibacterial compound, antibiotics, and biocides (Costa 2013). Efflux systems can remove non-antibiotic substrates such as dyes, detergents, heavy metals and organic solvents. In general, specific efflux pump genes can be found either on the chromosome or plasmids (Blanco *et al*, 2015; Li *et al*, 2015; Jang, 2016).

Major facilitator superfamily (MFS) comprises the secondary active transporter with single polypeptide chains containing 400 – 600 amino acids that crisscross the bacterial membrane and transport small solute across the membrane by using electrochemical gradients (Pao *et al.*, 1998). The genome of *S. aureus* contains more than 30 putative efflux pumps, a majority of which are of the Major Facilitator Superfamily (MFS) type (Lekshmi *et al*, 2017). MFS includes highly related secondary active and passive solute transporters, widely recognised as responsible for intrinsic and acquired antibiotic resistance in bacteria. Recently, a multidrug efflux pump LmrS is isolated from MRSA (Floyd *et al*, 2010). *LmrS* can confer high antibiotic resistance to

several antibiotics, the most prominent of them being lincomycin, fusidic acid, linezolid and erythromycin (Floyd *et al*, 2010; Kumar *et al*, 2016).

Efflux pumps not only help in the extrusion of antibiotics, heavy metals, organic pollutants, quorum sensing signals and toxic metabolites but also play important roles in the physiology of survival of the bacterium in unfavourable conditions (Handzlik *et al*, 2013). From food preservation and safety point of view, the physiology of efflux pumps in the survival of pathogenic or food poisoning bacteria in foods, processing environments and processing conditions assumes significance. In the context of efflux pumps as potential targets for inhibition for controlling pathogenic bacteria, it is important to understand the role of efflux pumps such as LmrS in the survival of *Staphylococcus aureus* in preserved foods.

With this background, the present study was performed with the following objectives:

- To study the survivability of methicillin-resistant *Staphylococcus aureus* (MRSA) in fish and fishery products.
- To study the role of *LmrS* in physiology and survivability of MRSA in fish.

APPENDIX

1. Baird Parker agar base (Hi-Media, Mumbai)

1.1. Composition (Ingredients – gms /Litre)

Ingredients		Gms / Litre
Tryptone	:	10.000
Peptone	:	5.000
Yeast extract	:	1.000
Glycine	:	12.000
Sodium pyruvate	:	10.000
Lithium chloride	:	5.000
Agar	:	20.000
Final pH (at 25°C)	:	7.0±0.2

63.0 grams of media was suspended in 950 ml distilled water and boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes cooled to 50°C and 50 ml concentrated Egg Yolk tellurite Emulsion (FD045) was aseptically added. The medium was mixed well and poured into sterile Petri plates.

2. Mannitol salt agar (Hi-Media, Mumbai)

2.1.Composition (Ingredients –gms /Litre)

Ingredients		Gms / Litre
Proteose peptone	:	10.000
Sodium chloride	:	75.000
D-Mannitol	:	10.000
Phenol red	:	0.025
Agar	:	15.000
Final pH (at 25°C)	:	7.4±0.2

111.02 grams suspended into 1000 ml of distilled water and boiled to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

3. Plate Count Agar (Hi-Media, Mumbai)

3.1. Composition (Ingredients –gms /Litre)

Ingredients		Gms / Litre
Casein enzymic hydrolysate	:	5.000
Yeast extract	:	2.500
Dextrose	:	1.000
Agar	:	9.000
Final pH (at 25°C)	:	7.0±0.2

17.5 grams suspended in 1000 ml of distilled water and heated to boiling to dissolve the medium completely. Sterilize was done by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

4. Luria Bertani Broth, Miller (Hi-Media, Mumbai)

4.1. Composition (Ingredients –gms /Litre)

Ingredients		Gms / Litre
Casein enzymic hydrolysate	:	10.000
Yeast extract	:	5.000
Sodium chloride	:	10.000
Final pH (at 25°C)	:	7.5±0.2

25 grams was suspended in 1000 ml of distilled water and heated to dissolve the medium completely. The medium was dispensed into the tube and sterilised by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

5. Nutrient agar

5.1. Composition (Ingredients –gms /Litre)

Ingredient		Gms / Litre
Peptone	:	5.000
Sodium chloride	:	5.000
HM peptone	:	1.500
Yeast extract	:	1.500
Agar	:	15.000
Final pH (at 25°C)	:	7.4±0.2

28 grams was suspended in 1000 ml of distilled water and heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mixed well and poured into sterile Petri plates.

6. Luria bertani agar (Hi-media, Mumbai)

6.1. Composition (Ingredients –gms /Litre)

Ingredients		Gms / Litre
Casein enzymic hydrolysate	:	10.000
Yeast extract	:	5.000
Sodium chloride	:	10.000
Agar	:	15.000
Final pH (at 25°C)	:	7.5±0.2

40 grams media was suspended into 1000 ml of distilled water and heated to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Dispense as desired. Mixed well and poured into sterile Petri plates.

7. Phosphate Buffered Saline, pH 7.2

7.1 Composition (Ingredients –gms /Litre)

Ingredients		Gms / Litre
Sodium chloride	:	8.500
Disodium hydrogen phosphate	:	1.910
Potassium dihydrogen phosphate	:	0.380
Final pH (at 25°C)	:	7.2±0.2

10.79 grams media suspended in 1000 ml of distilled water and heating to boiling to dissolve the medium completely. Sterilized by autoclaving at 10 lbs pressure (115°C) for 10 minutes.

LIST OF ACRONYM

%	Percentage
μl	Microliter
BPA	Baird Parker Agar Base
bp	Base pair
°C	Degree Celsius
cDNA	Complimentary DNA
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Etylenedimine trichloro- acetic acid
Fig	Figure
g.	Gram
h.	Hours
kb	Kilobase
LB	Luria-Bertani medium
min	minutes
ml	Millilitre
mM	Millimolar
OD	Optical density

PCR	Polymerase Chain Reaction
PBS	Phosphate buffer saline
RT- PCR	Real-Time PCR
rpm	Revolutions per minute
rRNA	Ribosomal RNA
Sec	Second
TE	Tris: EDTA
TPC	Total Plate Count
TAE buffer	Tris-acetate-EDTA

2. REVIEW OF LITERATURE

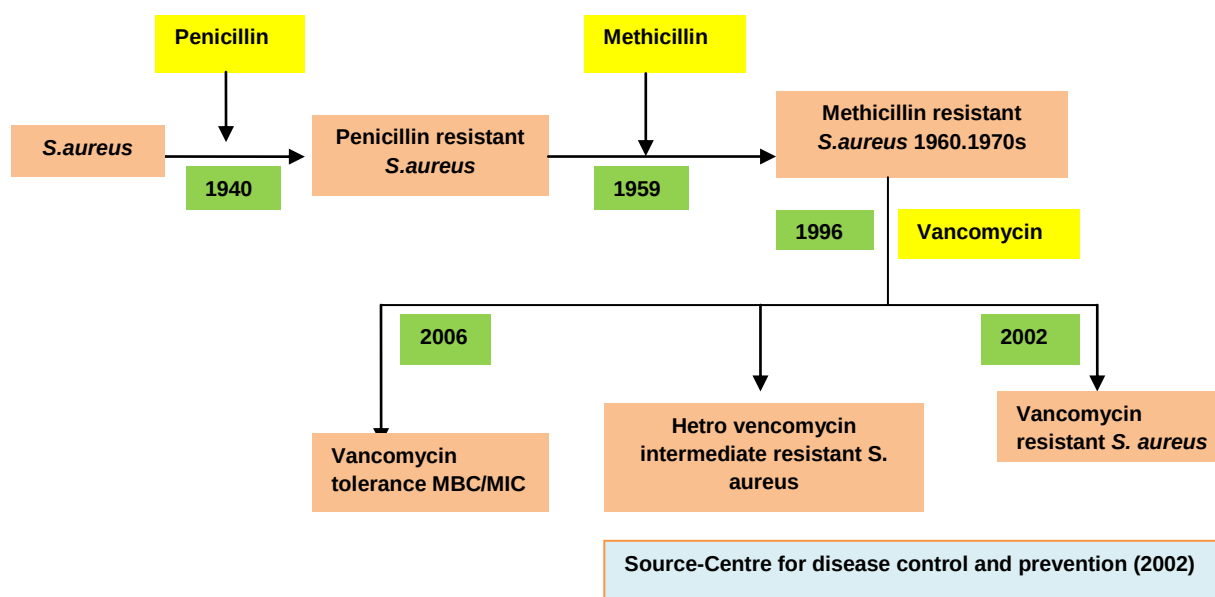
Staphylococcus aureus is a gram-positive, spherical shaped bacterium widely distributed in nature and found associated with humans and animals. It often asymptomatically colonizes the skin and mucous membranes of the nose, ear, throat, perineum, vagina and gastrointestinal tract and other parts of healthy humans and animals (Peacock *et al*, 2001; Bourgeois *et al*, 2010; Khan *et al*, 2015; Costa *et al*, 2013). Persistent colonization with *S. aureus* is observed in about 20-30% of the population, while the other 30% are transient carriers and approximately 50% are noncarriers (Otto *et al*, 2010). Colonization of *S. aureus* represents an increased risk of infection by providing a reservoir from which bacteria are introduced when the host defence is compromised (Pu *et al*, 2009; Brown *et al*, 2014). Infections importance of *S. aureus* are increasing the prevalence of antibiotic-resistant strains, the bacterium has become the most studied Staphylococcal species (Chambers, 2001; Kluytmans *et al*, 2005). *S. aureus* is the third largest cause of food-related illness throughout the world (Le Loir, 2003). *S. aureus* infection is a leading cause of staphylococcal bacteremia (skin and soft tissue infections, SSTIs) in adults and children worldwide (Saxena *et al*, 2006). Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) skin infections are occurring with increasing frequency in healthy individuals with no identified healthcare-associated risk factors (Lacey *et al*, 2016). *S. aureus* does not form spores, but it can cause contamination of food products during food preparation and processing (Kadariya *et al*, 2014).

2.1. History

Staphylococcus aureus is an opportunistic pathogen of humans, animals and other mammals. The bacterium was discovered in the 1880s. Sir Alexander Ogston, a Scottish surgeon, first showed in 1880 that some human pyogenic or performing diseases were associated with a cluster-forming micro-organism. He named the grape-like cluster of bacteria isolated from human abscesses as (*a staphyle*= bunch of grapes). The staphylococcal strains that produce golden yellow and white colonies on solid media were named *Staphylococcus aureus* and *S. albus* (later

renamed *S. epidermis*) respectively (Kloos, 1986). In 1959 methicillin was introduced to treat infections caused by penicillin-resistant *S. aureus*. Soon, *S. aureus* isolates that were resistant methicillin (methicillin-resistant *S. aureus*, MRSA) appeared in the UK and MRSA isolates were soon reported from other European countries, and later from Japan and Australia (Cuevase *et al*, 2007). MRSA emerged as a major health concern worldwide. MRSA is a problem in hospitals and is increasingly being recovered from nursing homes and the community. Many MRSA isolates are multiply resistant and are susceptible to glycopeptide antibiotics such as vancomycin. MRSA isolates which have decreased susceptibility to glycopeptides (glycopeptide intermediately susceptible *S. aureus*, GISA) also reported in the recent years, is a cause of great public health concern (Hiramatsu *et al*, 1997; Enright *et al*, 2002).

Evolution of drug target resistant in *Staphylococcus aureus*



2.2. General characteristics of *S. aureus*

Traditionally, staphylococci were divided into two groups by their ability to clot blood plasma (the coagulase reaction), i.e., coagulase positive (COPS) and

coagulase negative (CONS). The coagulase-positive staphylococci constitute the *Staphylococcus aureus* and *Staphylococcus epidermidis* (Arslan *et al*, 2009; Sastalla *et al*, 2017). Staphylococci are Gram-positive bacteria about 0.5 – 1.0 μm in diameter and grow in a spherical shape that occurs in tiny clusters resembling grapes and occasionally in short chains. The arrangement of the cocci helps to distinguish *Micrococci* and *Staphylococci* from *Streptococci*, which grow in chains. Further, micrococci and *Staphylococci* are catalase-positive and sugar fermentative, identifying them from the genus *Streptococci*, which are catalase-negative and non-fermentative. *S. aureus* is a facultative anaerobe which forms yellow colonies on rich medium. *S. aureus* has a relatively complex nutritional requirement. It requires an organic source of nitrogen, supplied by 5 to 12 essential amino acids, e.g., arginine, valine, and vitamin B, including thiamine and nicotinamide (Mah, 1967). It can grow at a temperature range of 15 to 45°C and resist high NaCl concentration of up to 15% (Harris *et al*, 2002). *S. aureus* produces different types of hemolysins that exhibit α -, β - and double ($\alpha+\beta$) hemolysis on blood agar plates. *S. aureus* expresses a wide array of cell-associated and secreted virulence factors. These properties make it a versatile pathogen which is responsible for a wide range of infections. The secreted factors include various enzymes, cytotoxins, exotoxins and exfoliative toxins (Dinges *et al*, 2000; Vitko *et al*, 2013).

2.3. Virulence factors of *S. aureus*

A broad range of infections caused in humans by *S. aureus* are attributed to the ability of this bacterium to express an array of elements which participate in the pathogenesis of infection, allowing this bacterium to adhere to surfaces or tissues, avoid the immune system, and cause harmful toxic effects to the host (Sandel *et al*, 2004; Costa *et al*, 2013). These factors are known as virulence determinants and can be divided into cell-surface-associated (adherence) and secreted (exotoxins) factors (Lowy, 1998; Dinges *et al*, 2000; Bien *et al*, 2011).

Table 1. Virulence factors of *Staphylococcus aureus* involved in the pathogenesis and respective putative functions

Factor	Putative function	References
Cell surface factors <i>Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)</i>		Speziale, (2009)
I. Staphylococcal protein A (SpA) and surface adhesion proteins	Block phagocytosis is determined by the presence of host antibody (Bind to IgG, interfering with opsonisation and phagocytosis).	Lacey <i>et al</i> , (2013); Kobayashi <i>et al</i> , (2013) Foster <i>et al</i> , (1998)
II. Fibronectin-binding proteins (FnbpA and FnbpB)	Attachment to fibronectin and clot plasma.	Pietrocola <i>et al</i> , (2016)
III. Collagen-binding protein	Adherence to collagenous tissues and cartilage.	Lacey <i>et al</i> , (2013)
IV. Clumping factor proteins (ClfA and ClfB)	Mediate clumping and adherence to fibrinogen in the presence of fibronectin.	Coast <i>et al</i> , (2013)
V. Capsular polysaccharides	Reduce phagocytosis by neutrophils; enhance bacterial colonisation and persistence on mucosal surfaces.	O'Riordan <i>et al</i> , 2004
VI. <i>Staphyloxanthin</i>	Resistance to neutrophil reactive oxidant-based phagocytosis, antioxidant.	Clauditz <i>et al</i> , (2006)
SECRETED FACTORS (<i>Superantigens</i>)		
vii. Staphylococcal enterotoxins (SEA, B, C, D, E, G and others.	Massive activation of T cells and antibody presenting cells.	Costa <i>et al</i> , (2013)
viii. Toxic shock syndrome toxin-1 (TSST-1).	Cause toxic shock syndrome.	Otto, (2014)

Cytolytic toxins Cytolysins		
ix. α -hemolysin	Induce lysis on a broad spectrum of cells, mainly platelets and monocytes.	Kong <i>et al</i> , 2016, Memestrina <i>et al</i> , 2001
x. β -hemolysin	Hydrolysis of sphingomyelin of the plasmatic membrane of monocytes, erythrocytes, neutrophils and lymphocytes.	Costa <i>et al</i> , (2013)
xi. γ -hemolysin	Induce lysis on erythrocytes and Leukocytes.	Lacey <i>et al</i> , (2013)
xii. Panton-Valentine leukocidin (PVL)	Induce lysis on leukocytes, massive tissue inflammation and necrosis.	Francis <i>et al</i> , 2005 Holzinger <i>et al</i> , 2012
Various exoenzymes		
xiii. Lipases	Inactivate fatty acids nucleases cleave nucleic acids.	El-baz <i>et al</i> , (2016)
xiv. Proteases	Inactivate fatty acids Cleave nucleic acids.	Lacey <i>et al</i> , (2016)
xv. Hyaluronidase	Degrade hyaluronic acid.	Ibberson, (2015)
xvi. Staphylokinase (SAK)	Activate plasminogen; inactivate antimicrobial peptides.	Bokarewa <i>et al</i> , (2006)
Miscellaneous proteins		
xvii. Staphylococcal complement inhibitor (SCIN)	Inhibit complement activation.	Costa <i>et al</i> , (2013)
xviii. Extracellular fibrinogen binding protein (Efb).	Inhibit chemotaxis and activation of Neutrophils.	Rooijackers <i>et al</i> , (2007)
xix. Chemotaxis inhibitory protein of <i>S. aureus</i> (CHIPS).	Inhibit chemotaxis of neutrophils.	de Haas <i>et al</i> , (2004)
xx. Formyl peptide receptor-like one inhibitory protein(FLIPr).	Inhibit neutrophil migration.	Prat <i>et al</i> , (2006)

2.4. Infections caused by *S. aureus*

S. aureus is accountable for various diseases caused by direct infection including mild skin infections (impetigo, folliculitis etc.), invasive diseases (wound infections, osteomyelitis, bacteremia with metastatic complications, etc.) (O'Riordan *et al*, 2004). Indirect production of toxins in food gives rise to scaled skin syndrome, toxic shock syndrome (TSS) leading to fever, sickness and severe conditions may cause death. *S. aureus* also causes mastitis (infection of the mammary glands) in cows, sheep, and goats (Lowy, 1998; Arslan, 2009; Ortega *et al*, 2010).

Some enterotoxigenic strains of *S. aureus* are responsible for causing severe food poisoning by heat-stable staphylococcal enterotoxins (SEs) which leads to gastroenteritis staphyloenterotoxiosis or staphyloenterotoxemia within 1 to 7 h after consumption of contaminated food (Le Loir, 2003). TSST-1 was the first toxin shown to be involved in toxic shock syndrome. Enterotoxins and toxic shock syndrome toxin 1 (TSST-1) are members of the pyrogenic toxin superantigen (PTSAg) family (Arfatahery *et al*, 2016).

2.5. Nature and structure of Staphylococcus enterotoxins (SEs)

Staphylococcal enterotoxins (SEs) are broadly classified as superantigens, and can stimulate large populations of T cells (~20–30%) leading to the production of a cytokine bolus (Choi *et al*, 1989.) At least 20 serologically distinct staphylococcal superantigens have been described that include SEs A through V and toxic shock syndrome toxin-1 (TSST-1). SEA, SED, and SEE share 70–90% sequence homology, while only 40–60% with SEB, SEC, and TSST-1 (Pinchuk *et al*, 2010). Their mature length is approximately 220–240 amino acids, depending on the toxin, and their molecular size is on average ~25 kD and have significant sequence variability, but when folded have similar three-dimensional structures (Al-Daccak *et al* 1998; Schlievert *et al*, 1995).

The three-dimensional structure for multiple SEs has been determined by crystallography and found that they are large elliptical in configuration and have two major unequal domains composed mostly of β strands and a few α -helices (Singh *et al*,

1994; Sundström *et al*, 1996; Pinchuk *et al*, 2010). The two domains are separated by a shallow cavity. The larger of the two domains contains both the amino and carboxyl terminal. Mutational analysis of both SEA and SEB implicated this cavity in the binding to T cell receptors (TcR) (Garcia *et al*, 1998; Antonsson *et al*, 1997). Another region on SEA identified by mutational analysis to interact with the TcR V β 7 and 8.1 is tyrosine 66, while a stretch of amino acids from 45 to 58 on SEB was found to be involved in the binding to class II major histocompatibility complex (MHC) molecules that are expressed by antigen presenting cells (APC) (Kappler *et al*, 1998 Pinchuk *et al*. 2010)

2.6. Staphylococcal food poisoning (SFP)

Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases in the world following the ingestion of milk and cream, cream-filled pastries, butter, ham, cheeses, sausages, canned meat, salads, cooked meals and sandwich fillings which contains staphylococcal enterotoxins (SEs) (Schmid *et al*, 2009; Normanno *et al*, 2007; Ortega *et al*, 2010). These Staphylococcal enterotoxins are low molecular weight proteins (MW 26,900–29,600), which are divided mainly into different serotypes: SEA, SEB, SEC1, 2, 3, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEM, SEN, SEO, SEP, SEQ, SER, SES, SET, SEU1, 2 and SEV (Balaban & Rasooly 2000; Le Loir 2003; Hennekinne *et al*, 2012). Production of SEs by enterotoxigenic strains of coagulase-positive *Staphylococci* mainly *S. aureus* and very occasionally by other *Staphylococci* species such as *Staphylococcus intermedius* (Sandel *et al*, 2004; Kerouanton *et al*, 2007). *Staphylococcal* food poisoning occurs when food consumed contains SE produced by *S. aureus*. The worldwide outbreaks of SFPs are due to the characteristics and behaviour of *S. aureus* in the food environment, and the toxinogenic characteristics and conditions of SEs, in food (Ortega *et al*, 2010).

Food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands are regarded as the primary sources of food contamination *via* direct contact or through respiratory secretions (Argudin *et al*, 2010). Symptoms of SFP include rapid onset (within 1–6 h) of nausea, vomiting, diarrhoea and abdominal

cramps. These symptoms resolve within 24 – 48 h but the illness may remain for 7–10 days. A human feeding trial in the 1960s demonstrated that 20–25 µg (0.4 µg/kg body weight) of enterotoxin B caused illness (Raj and Bergdoll, 1969). Recently it has been reported that less than 1.0 µg of SE are sufficient to cause staphylococcal food poisoning (FDA 2012). In fact, evidence from outbreaks indicates that ingestion of less than 200 ng of the SEA is enough to cause illness in susceptible individuals (Evenson *et al*, 1988). *Staphylococcal* enterotoxins are resistant to environmental conditions (freezing, drying, heat treatment and low pH) that easily destroy the enterotoxin-producing strain (Hennekinne *et al*, 2012; Hu *et al*, 2014).

Enterotoxin gene locations are very numerous inside of the bacterial cell (Argudin *et al*, 2010). They can be carried by plasmids (SEB, SED, SEJ, SER, SES, SET) (Shalita *et al*, 1977; Ono *et al*, 2008), phage (SEA, and SEE) (Coleman *et al*, 1989) or by genomic islands (SEB, SEC, SEG, SHE, SEI, SEK, SEL, SEM, SEN, SEO, SEP, and SEQ) (Hennekinne *et al*, 2010).

2.7. Major outbreak by *Staphylococcus* food poisoning

Table 2. Staphylococcal Enterotoxins (SEs) outbreak in the world

Year	Location	Cause	No. Of cases	Reference
2000	Osaka, Japan	Low-fat milk	13 420	Asao <i>et al</i> , (2003)
2006	France	Coconut pearls (Chinese dessert)	17	Hennekinne <i>et al</i> , 2012
2007	Austria	Milk, cacao milk, vanilla milk	166	Schmid <i>et al</i> , (2009)
2008	French district	Pasta salad	100	Hennekinne <i>et al</i> , 2012
2009	Nagoya university festival, Japan	Crepes	75	Kitamoto <i>et al</i> , (2009)
2009	Various districts, of France	Raw milk cheese	23	Ostyn <i>et al</i> , (2010)
2012	United state	Perlo (a chicken, sausage, and rice dish)	22	https://www.cdc.gov
2014	New South Wales, Australia,	Pickled grilled salmon	22	https://www.ncbi.nlm.nih.gov

2.8. Methicillin-resistant *S. aureus* (MRSA)

In the late 1940s and throughout 1950s, *S. aureus* developed resistance towards penicillin (Shaffer, 2013). William M.M. Kirby first noted Penicillin-resistant *S. aureus* at Stanford University School of Medicine in 1944. Methicillin, which was introduced in 1961 as another form of penicillin to counter the increasing problem of penicillin-resistant *S. aureus*. Methicillin was the first of the semisynthetic penicillinase-

resistant penicillins. Reports of methicillin-resistant isolates rapidly followed its introduction. Methicillin was the most common type of antibiotic used to treat *S. aureus* infections, but the first strains of *S. aureus* bacteria that resisted methicillin were discovered by British scientists in 1961 (Report of National Institute of allergy and infectious diseases 2016). The first reported human case of MRSA in the United States was in 1968 (Lowy, 2003; Barrett, 1968; Shaffer *et al*, 2013). Methicillin resistance is an indicator of *S. aureus* being resistant to several other antibiotics and is a significant health risk in the hospitals, community and the livestock. MRSA strains that are resistant to monovalent cationic antimicrobials, bivalent quaternary ammonium compounds, macrolides, quinolones, tetracycline, and beta-lactams are now widespread. Methicillin-resistant *S. aureus* (MRSA) is well known as an agent of nosocomial infections, and MRSA that is also resistant to vancomycin (VRSA) has been reported from all over the world (Lowy *et al*, 1998).

Methicillin-resistant *S. aureus* was first reported as hospital-associated (HA-MRSA) (Deresinski, 2005). Methicillin-resistance is attributed to the presence of *mecA* gene by horizontal gene transfer (Bloemendaal, 2010; Cafini, 2017). The *mecA* gene is located on complex mobile genetic elements, known as SCCmec ("staphylococcal cassette chromosome" or "staphylococcal chromosomal cassette" (Wielders *et al*, 2002; Raygada *et al*, 2009). *mecA* gene encodes a transpeptidase, designated penicillin-binding protein 2 (PBP2 or PBP2a) involved in cell-wall peptidoglycan synthesis. Staphylococcal PBPs produce a tightly cross-linked peptidoglycan structure, resulting in a pressure durable cell wall (Hiramatsu *et al*, 2002). Beta-lactam antibiotics bind the intrinsic staphylococcal PBPs, inhibiting their ability to crosslink the D-alanyl-D-alanine residue of one peptidoglycan monomer to the pentaglycine chain of another (Sancak *et al*, 2000; Hiramatsu *et al*, 2002). Structural alterations in the PBP-2 result in the inability of the antibiotic to bind to the protein resulting in the resistance. In addition to *mecA*, SCCmec elements comprise recombinase genes, regulatory factors and, variably, additional genes encoding resistance to other antimicrobials, such as aminoglycosides or macrolides, and too heavy metal ions. SCCmec types I, II and III (or 1B, 2A, 3A, are typically restricted to MRSA strains associated with healthcare infections and are not found widely among

the healthy population (Deurenberg *et al*, 2008). These strains, which were initially known as "Epidemic MRSA" (EMRSA), are now frequently referred to as "hospital-acquired" or "healthcare-associated" MRSA (HA-MRSA). MRSA isolates that have been decreased susceptibility to glycopeptides (glycopeptide intermediately susceptible *S. aureus*, (GISA) reported in recent years, are a cause of significant public health concern (Enright *et al*, 2002 ; Chambers *et al*, 2009).

2.9. Association of MRSA with hospital, community, and livestock

Initially, MRSA was restricted to hospitals and known as hospital-associated MRSA (HA-MRSA). MRSA infections may occur outside of hospitals, caused by community-associated MRSA (CA-MRSA) (Huijsdens *et al*, 2006; Venter *et al*. 2017). MRSA infections in the community can be caused by livestock-associated MRSA (LA-MRSA). LA-MRSA is initially associated with livestock and differs from genotypic HA-MRSA and genotypic CA-MRSA in its genomic traits (O'Mahony *et al*, 2005; Cuny, 2015). MRSA was found from milk in Belgian cows with mastitis (Devriese *et al*, 1975). MRSA has since been reported in many diverse species, including dogs cats, sheep, chickens, horses, rabbits, seals, psittacine birds, and one turtle, bat, guinea pig and chinchilla (Baptiste *et al*, 2005; Walther *et al*, 2008). *S. aureus* was already a major problem in dairy cows, where it causes mastitis, toxins productions, and infections in chickens have also caused problems (Benkerroum, 2018; Quddoumi *et al*, 2006; Meemken *et al*, 2010). Pigs are often carriers and are only rarely infected. In chickens, several disease manifestations have been described, such as comb necrosis, bacterial chondronecrosis, which is a cause of leg weakness or lameness, and septicaemia. These diseases may affect a significant proportion of a flock (Fluit *et al*, 2012; McNamee *et al*, 2000). The strain of MRSA which is isolated from LA-MRSA is the major cause of infection in humans (Kitai *et al*, 2005; Fluit *et al*, 2012).

2.10. Mechanisms of antibiotic resistance in bacteria

The mechanisms of antibiotic resistance can be divided into the following categories.

- Drug target modification
- Bacterial enzyme inactivate or degradation of the antibiotics
- Biofilm formation
- Reduce the bacterial cell membrane permeability
- Active efflux of antimicrobials from bacteria pathogen (Costa *et al*, 2013).

In all of these cases, an isolate of bacteria is carrying resistance-conferring mutations selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive and grow (Walsh, 2000; Tenover, 2006). The acquired resistance that develops due to chromosomal mutation and selection is termed vertical evolution (Nikaido, 2009). Bacteria also build resilience through the acquisition of new genetic materials from other resistant organisms, which is termed parallel development and may occur between strains of the same species or between different bacterial species or genera (Handzlik *et al*, 2013; Aminov, 2011; Nikaido, 1994). Mechanisms of genetic exchange include conjugation, transduction, and transformation (Du *et al*, 2015; Tenover, 2006; Puglia *et al*, 2017).

2.10.1. Drug target modification or altering the target structure

One of the most common strategies is the alteration of the target sites (sites of action) of antibiotics, often by a mutation that alters the drug binding site, permanently inactivating the antibiotics and allowing the bacterial pathogen to grow in the presence of the antimicrobial agent. Alteration of cellular drug target proteins through various modifications so that the drug no longer efficiently binds to its destination. Such objectives in a bacterial cell include the ribosomal protein subunits, nucleic acids, metabolic enzymes, and the bacterial cell wall components, e.g., change in penicillin-binding protein 2b in pneumococci, which results in penicillin resistance. (Costa *et al*. 2013; Kumar *et al*, 2013; Andersen *et al*, 2015, Leclercq *et al*, 1991)

2.10.2. Enzymatic inactivation or degradation of the antibiotics

In this particular mechanism, the antibacterial drug will be enzymatically altered to an inactive state such that the new drug metabolite cannot inhibit or kill the bacterium and also reduce the cell membrane permeability, e.g., erythromycin ribosomal methylase in staphylococci (De Pascale *et al*, 2010; Thakker-Varia, 1985). Most of the antibiotics have hydrolytically susceptible chemical bonds (e.g., esters and amides); and several enzymes have evolved that cleave these weak bonds and as a result, produce an inactive form of the antimicrobial agent thus providing a means for the bacteria to grow. Mechanism of antibiotic inactivation involves enzyme-mediated structural alteration of the drug via transfer of a functional group such as an acyl, ribosyl, phosphoryl or thiol group of beta-lactam ring (O Gutkind, 2013). The reaction is irreversible, and the modified antibiotic is unable to bind to the target due to the resultant change in the structure (Kumar *et al*, 2013; Anderson *et al*, 2015; Blair *et al*, 2015).

2.10.3. Alteration of bacterial cell membrane permeability

Bacteria are unicellular organisms, and their cytoplasm is separated from the external environment by the cytoplasmic membrane. The significant permeability barrier in any layer is the lipid bilayer structure, and its barrier property is inversely correlated with its fluidity. Mechanism of reduction in cell membrane permeability is due to the modification of the outer membrane lipid barrier and the inability of antimicrobial agent to gain entry into the cell where the drug targets are located and diminish the protein/ porin channels to decrease drug entry. Such a drug-resistant bacterium might have opted to cut the expression of a putative drug transport system in the membrane thus reducing cellular drug entry (Kumar *et al*, 2013; Anderson *et al*, 2015; Costa *et al*, 2013).

2.10.4. Biofilm formation

Bacteria in biofilms are attached to a solid surface, either biological or non-biological surfaces (Aminov, 2011). Biofilms are covered by thick and slimy matrix made up bacterial exopolysaccharide. A biofilm may contain a single species of bacteria, or more than one species may aggregate to form a biofilm, fungi including yeasts are occasionally involved (Kelechi 2015). Biofilm formation represents a protective mode of growth that allows microorganisms to survive in hostile environments (Davies, 1998). Biofilm is responsible for persistent chronic infections, due to their inherent resistance to antimicrobial agents (Tabatabaei and Sohrabi, 2017). Biofilms are important to cause infections in human that are persistent and difficult to treat, such as *S. epidermidis* and *S. aureus* infections of central venous catheters, eye infections such as occur with contact lenses and intraocular lenses, in dental plaque, and with *Pseudomonas aeruginosa* airway infections in cystic fibrosis patients (Kelechi *et al*, 2015). Biofilm production occurs in many loci, wound, water environments, and food processing surfaces etc. As such, microorganisms that are found in biofilms are protected from the entry of various types of antimicrobial agents. In the mechanism of biofilm formation, mode of growth bacteria conferring less susceptibility towards drugs could be due to a protective mutation in plasmids or mobile genetic elements or horizontal gene transfer among bacteria (Stewart, 2001) For example, enzymes produced by bacterial cells in biofilms inactivate antibiotic action by degrading or breaking chemical bonds and increased expression of an efflux pump that has a broad range of substrate. Some other mechanisms like Poor diffusion of antibiotics through the biofilm polysaccharide matrix and phenotypic changes in biofilm-forming bacteria are also reportedly responsible for the antimicrobial resistance in biofilm structures (Kumar *et al*, 2013; Andersen *et al*, 2015).

2.10.5. Active efflux of antimicrobials

Bacteria show resistance to antibacterial agents and other toxic compounds by a mechanism known as active efflux, where the integral membrane transporters known as drug efflux pumps, prevent the accumulation of drugs inside the

bacterial cells (Andersen *et al*, 2015). Efflux pumps are membrane proteins which have the function of detoxifying cells by expelling noxious molecules. Genes and proteins of efflux pump are present in both antibiotic-susceptible and antibiotic-resistant bacteria (Piddock, 2006; Nikaido, 1994). The extrusion of antimicrobial compounds, such as biocides and antibiotics, is considered to be an accidental function of such efflux systems. These specific efflux pumps genes can be found either in the chromosome or transmissible genetic elements such as plasmids. Multidrug efflux pumps are mainly located on the chromosomes, except QacA/B and Smr, which only have been described in plasmids (Costa *et al*, 2013; Kumar *et al*, 2005).

Efflux pumps consist of hydrophobic proteins that are capable of exporting specific drug classes or structurally diverse compounds and toxins utilising ATP or ion gradients as a source of energy (Schindler *et al*, 2013). Efflux protein is categorised by energy requirement, structural and subcellular organisation difference. Bacterial efflux systems can be either specific, extruding only one antibiotic or class of antibiotics, or be capable of ejecting several types of antimicrobial compounds, being designated multidrug resistance efflux (MDR) pumps (Kumar *et al*, 2005; Costa *et al*, 2012).

2.11. Classification of multidrug efflux pumps

MDR efflux systems are classified into five families according to their energy requirements and structure namely, the

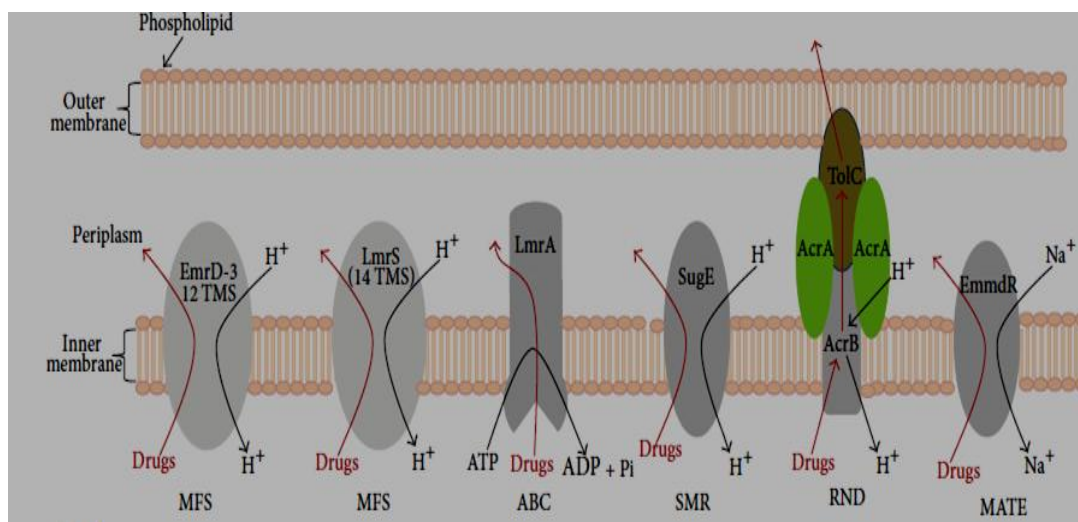
- I. Major facilitator superfamily (MFS)
- II. Small multidrug resistance (SMR) family
- III. Multidrug and toxic compound extrusion (MATE) family
- IV. Resistance-nodulation-cell division (RND) superfamily
- V. Adenosine-triphosphate (ATP)-binding cassette (ABC) superfamily

Table 3. Classification of MDR efflux systems in five families according to their energy requirements and structure

Name of the multidrug-resistant (MDR) efflux pump	Source of energy
i. Major facilitator superfamily (MFS)	Proton motive force
ii. Small multidrug resistance (SMR) family	Proton motive force
iii. Resistance-nodulation-cell division (RND) superfamily	Proton motive force
iv. Multidrug and toxic compound extrusion (MATE) family	Sodium membrane gradient
v. Adenosine-triphosphate (ATP)-binding cassette (ABC) superfamily	ATP

Source. (Webber et al, 2003; Costa et al, 2013; (Kumar et al, 2012).

The third category of drug pumps, called the phosphotransferase system (PTS), catalyzes the transport of drug with concomitant phosphorylation of the drug, usually for cellular entry of the drug substrate. Efflux systems of the four families: MFS, ABC, SMR and MATE are widely distributed in both Gram-positive and negative bacteria. Efflux pumps are either single-component transporters or multiple-component systems containing not only an inner membrane transporter but also an outer membrane channel and a periplasmic adaptor protein (Sun et al, 2014; Higgins et al, 2004).



Source. (Kumar *et al*, 2013)

Table 4. Different type of efflux pumps in Methicillin-resistant *S. aureus* (MRSA)

Family	Efflux pump	Substrate specificity	References
Chromosomally encoded efflux pump 	NorA, NorB, NorC	- Fluroquinolones, - Chloramphenicol	Kumar <i>et al</i> ,
	MdeA	- Fluroquinolones - Quaternary ammonium compound (benzalkonium chloride, dequalinium)	Costa <i>et al</i> ,
	Lmrs	- Linezolid, - Phenicol (chloramphenicol, florfenicol) - quaternary ammonium compounds (tetraphenylphosphonium) - detergents- sodium dodecyl sulphate - dyes – ethidium bromide	Kumar <i>et al</i> ,
Major facilitator family(MFS) 	QacA and QacB	- Quaternary ammonium compounds-dequalinium, benzalkonium chloride - Dyes- sodium dodecyl	Costa <i>et al</i> ,

2.12. Major facilitator superfamily

The major facilitator superfamily (MFS) of transporters comprised uniporters, symporters, antiporters and called as uniporter-symporter-antiporter (USA) family. Henderson and coworkers discovered the MFS (Kumar *et al*, 2013). The major facilitator superfamily (MFS) is the most significant known superfamily of secondary carriers found in the biosphere. It is ubiquitously distributed throughout virtually all currently recognised organismal phyla. The MFS is grouped further into 76 subfamilies based on phylogenetic analysis, substrate specificity and operational mechanism, in the Transporter Classification Database (TCDB) (Saier *et al*, 1999; Lekshmi *et al*, 2017). The MFS efflux proteins can be divided into two distinct clusters with either 12 or 14 transmembrane segments. The efflux proteins of MFS are of the antiporter group and genes encoding these efflux pumps are mostly chromosomal, but some are plasmid-borne (Reddy *et al*, 2012; Kumar *et al*, 2013; Lekshmi *et al*, 2017; Varela *et al*, 2017).

2.12.1. *NorA*, *NorB* and *NorC* efflux proteins

NorA was the first efflux pump to be discovered in a fluoroquinolone-resistant *S. aureus* was isolated from a Japanese hospital in 1986 (Costa *et al*, 2013). NorA, together with QacA and QacB, constitute the best-characterised efflux pumps from *S. aureus* (Andersen *et al*, 2015). NorA has 388 amino acids that fold into 12 transmembrane helices with a molecular weight of 42.2 kDa (Costa *et al*, 2013). NorA is chromosomally encoded and shares 40% amino acid identity with Bmr efflux pump of *Bacillus subtilis* (Andersen *et al*, 2015). It was initially thought that fluoroquinolones were the only substrates of NorA efflux pump, but later works showed that NorA extrudes a variety of non-fluoroquinolone antibiotics such as tetracycline and an array of chemicals including ethidium bromide, cetrimide and tetraphenylphosphonium (TPP). NorB is a chromosomally encoded efflux pump that contains 463 residues with 12 transmembrane segments. The *norB* gene is 1392 bp long and encodes a 49 kDa protein (Costa *et al*, 2013; Lekshmi *et al*, 2017). The chromosomal gene *norC* codifies the efflux pump NorC. It is a 462 amino acid protein with 12 transmembrane segments

that belong to the MFS and shares 61% identity with NorB (Andersen *et al*, 2015; Costa *et al*, 2013; Lekshmi *et al*, 2017).

2.12.2. Qac group of efflux pumps

The Qac group of efflux pumps consists of QacA, QacB, QacG, QacH and QacJ transporters (Lekshmi *et al*, 2017). QacA and QacB are MFS transporters, both 514 amino acids long and 55 kDa in size, and carriers rely on the proton motive force for drug extrusion from the bacterial cell (Andersen *et al*, 2015) QacA was the first 14 α -helical transmembrane antimicrobial transport protein to be studied by using fusion proteins with alkaline phosphatase and β -galactosidase reporter enzymes. The *qacA* and *qacB* genes are similar in their DNA sequences, differing by only 7 nucleotides. Thus, it is not surprising that their substrates are similar but not identical (Paulsen *et al*, 1996; Andersen *et al*, 2015).

2.12.3. TetA(K) and Tet38 efflux pumps

TetA(K), first reported in a strain of *S. aureus*, has 459 amino acids that form 12 transmembrane segments (Lekshmi *et al*, 2017). The TetA(K) efflux pump is characterised by its ability to confer high resistance to the tetracycline class of antibiotics. TetA(K), or Tet(K), is a plasmid-encoded efflux pump that acts as an $\text{Na}^+ (\text{K}^+) / \text{H}^+$ antiporter (Krulwich *et al*, 2001; Chopra *et al*, 2001). It consists of 459 amino acids having a similarity of 60% with TetA(L). TetA(K), a 7 kDa protein consisting of 14 transmembrane segments, belongs to the MFS of transporters (Andersen *et al*, 2015). TetA(K) confers high levels of resistance to oxytetracycline, tetracycline and chlortetracycline. However, less resistance is seen for antibiotics like minocycline, 6-dimethyl-6-oxytetracycline and doxycycline (Roberts, 2005; Costa *et al*, 2013; Andersen *et al*, 2015; Lekshmi *et al*, 2018).

2.12.4. SdrM Multidrug efflux Pump

SdrM is a multidrug efflux pump with 14 predicted transmembrane segments and belongs to MFS. SdrM is 23% identical and 68% similar to NorB; likewise, SdrM is 21% identical and 63% similar to QacA (Costa *et al*, 2013). MgrA also regulates its expression, and mutation of the mgrA gene may cause enhanced expression from the sdrM (*Staphylococcus* drug resistance) gene leading to the emergence of more resistant strains (Costa *et al*, 2013). SdrM drug transporter is responsible resistances to antimicrobial agents like acriflavine, ethidium bromide, fluoroquinolone, and norfloxacin are observed, With 447 amino acids and 14 predicted transmembrane segments (Andersen *et al*, 2015; Lekshmi *et al*, 2017).

2.12.5. MdeA efflux pump

The MdeA protein is a member of the MFS family of efflux pumps made up of 479 amino acids that form 14 transmembrane helices 1437 bp mdeA gene. MdeA confers resistance on *S. aureus* to a range of quaternary ammonium compounds and antibiotics, but not fluoroquinolones. MdeA is a 52-kDa protein with 14 predicted transmembrane segments (Huang *et al*, 2004). This 52 kDa protein effluxes fluoroquinolones, although with low affinity. Efflux with low levels of sequence similarity with MdeA includes QacA (23% similarity), EmrB of *E. coli*, LmrB of *B. subtilis* and FarB of *Neisseria gonorrhoeae*. Mutations in the promoter region of MdeA result in the overexpression of the pump (Lekshmi *et al*, 2017).

2.12.6. Lmrs efflux pump of *Staphylococcus aureus*

Especially multidrug efflux pumps of the major facilitator superfamily such as LmrS, NorA, MdeA, MefA, QacA, SdrM, TetA(K) and Tet38 are expressed in *S. aureus* variants and represent significant resistance mechanisms (Costa *et al*, 2013; Andersen *et al.*, 2015; Kumar and Varela, 2012). The multidrug efflux pump LmrS (lincomycin multidrug resistance protein of *S. aureus*) was first cloned from MRSA clinical samples and contributes to multiple drug resistance. The *lmrS* gene (GenBank:

AEG25669.1) is 1443 nucleotides long and encodes a mostly hydrophobic integral membrane protein of 480 amino acids with a molecular mass of 47 and 14 kDa predicted transmembrane domains. LmrS shares identity with other lincomycin resistance efflux pumps from *Bacillus* spp., *Lactobacillus* spp., and *Listeria* spp. As a second active drug/H⁺ antiporter, LmrS actively extrudes structurally different compounds including lincomycin, linezolid, kanamycin, trimethoprim, florfenicol, chloramphenicol, erythromycin, streptomycin, and tetraphenylphosphonium chloride (TPCL), thus conferring bacterial resistance to multiple antimicrobial agents (Floyd *et al*, 2010). Thus, LmrS from *S. aureus* is a good target candidate for modulation of bacterial antimicrobial strength, to regain the effectiveness of chemotherapeutic agents that are compromised by bacterial multidrug efflux systems (Kumar *et al*, 2016). The secondary structure of LmrS has 14 predicted transmembrane domains. Linezolid is vital in the treatment of methicillin-resistant *S. aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *Enterococcus* spp. (VRE). *S. aureus* resistance to linezolid has been reported previously. Linezolid resistance in Gram-positive bacteria has been attributed to a mutation at position 2576 of 23S rRNA. They suggested that the efflux protein LmrS is an essential determinant of antimicrobial resistance in *S. aureus* (Kakarla *et al*, 2017).

2.13. Role of an efflux pump in bacterial survival and pathogenicity

The efflux pump typically export several unrelated substances, including molecules produced by the host organism (such as bile), indicating that these systems might have a role in allowing bacteria to survive in their ecological niche and physiological niches. Certain classes of efflux pump not only confer resistance to drugs used in therapy but also have a role in bacterial pathogenicities such as in the colonization and the survival of bacteria in the host (Blanco *et al*, 2016). Some bacterial efflux pumps export not only antibiotics and other substances such as dyes and detergents, but also host-derived antimicrobial agents (Piddock, 2006). Efflux pumps play a general role of detoxification in several bacterial physiological processes. Exploring the regulation of efflux pump genes as well as their specific physiological

functions will advance our understanding of the naturally or physiologically originated drug resistance which frequently occurs in nature (Sun *et al*, 2014).

2.14. The occurrence of *S. aureus* in seafood

The problem of seafood contamination with pathogenic microorganisms has become a critical food safety issue in developing and highly populous countries of the world. Fresh fish and seafood are free from *S. aureus*. Hence their presence on fish is a clear indication of secondary contamination 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*. Many other 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 (Kumar *et al*, 2016). Past studies have reported the presence of antibiotic-resistant *S. aureus* in fresh seafood, ready-to-eat fish, seafood products and also in cultured fish, antimicrobial resistance and sensitivity in bacteria have been considered as a significant problem in Public Health. Acco *et al*, 2003; Kumar *et al*, 2017) Showed that 70% of the *S. aureus* strains isolated from food handlers were resistant to penicillin. When enterotoxigenic strains are present in unheated raw materials, the growth of *S. aureus* may not control, and its contamination can induce food poisoning outbreak (Hammand *et al*, 2012; Park *et al*, 2014).

In one of the earliest studies on *S. aureus* in seafood, Iyer and Shrivastava (1988) detected coagulase-positive staphylococci in 38% of the cooked frozen shrimps. Simon *et al*, (2007) studied the occurrence of enterotoxigenic *S. aureus* in fish, fishery products and in fish processing factory workers in Cochin. These investigators found that of 168 fishery products, 21 (17%) and 87 samples from fish processing factory workers 54 (62%) were found positive for enterotoxigenic *S. aureus*.

Kumar *et al*, (2016) studied the occurrence of methicillin-resistant staphylococci in fresh seafood, seafood products and related samples in Mumbai region. Coagulase-positive *S. aureus* was isolated from 15.78% of the seafood

samples. In this study, the *mecA* gene was detected by PCR in 10 MR-CoNS and one MRSA strain. The *lmrS* gene, which codes for a multidrug efflux pump LmrS, was detected only in coagulase-positive isolates (Kumar *et al*, 2016).

Ozturk *et al*, (2018) reported the prevalence of coagulase-positive *S. aureus* in raw and ready-to-eat seafood samples in Izmir (Turkey) and determined the antibiotic resistance of the isolates. Among 50 samples of fresh and ready-to-use seafood analysed, coagulase-positive *S. aureus* was detected in 24 (58%) raw and 26 (46%) ready-to-eat seafood. While all *S. aureus* isolates were susceptible to ceftazidime, clindamycin, gentamycin, nitrofurantoin, ofloxacin, oxacillin, rifampicin, vancomycin and amikacin, the strains were resistance to amoxicillin-clavulanic acid and ciprofloxacin (7.7%), methicillin (30.8%), cephalotone (3.8%), penicillin (80.8%), linezolid (19.2%).

Murugadas *et al*, (2017) conducted a pilot study to understand and identify the possible source of contamination from a landing centre to retail fish market and local spread of MRSA clones in Kerala, India. 17 samples which include seafood and fishery environment samples from the retail fish market and a landing centre were collected to identify the source of contamination of MRSA. MRSA strains were isolated from 35.2% to 23.5% of samples. 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 sources of contamination during handling at the landing centre.

2.15.Survival of *Staphylococcus aureus* in a different storage conditions

2.15.1. Survival of *Staphylococcus aureus* in a low-temperature storage condition

Low temperature can change the response of a microorganism either directly or indirectly. Direct effects include decreased growth rate, alteration of cell composition, enzyme activities and differential nutritional requirements. Indirect effects are generally observed on the solubility of solute molecules, diffusion of nutrients,

osmotic impact on membranes and cell density (Beleneva, 2011; Tanka *et al*, 1999). As the temperature falls, the lag phase that precedes growth extends, leading to a decrease in the growth rate and the final cell number (Nedwell, 1999; Beales, 2004). *S. aureus* is resistant to freezing and survives well in food stored below -20° C. However, viability is reduced at temperatures of -10 to 0°C. Some cold shock proteins may be produced when low temperature challenges an organism (Berry *et al*, 1984). Desaturase enzymes associated with modification of the cell membrane in response to low temperature (Nedwell *et al*, 1999). Some studies indicate that *S. aureus* increased in glycolytic enzyme production and changed the lipid components of its membrane to acclimate to a cold environment (Moon *et al*, 2017).

2.15.2. Survival of *S. aureus* in dry fish

Low-moisture foods are commonly considered as safe and storable products because of their low water activity ($a_w < 0.6$). Most microorganisms do not grow but can survive in food with this level of water activity. Preservation by drying is achieved by the extraction or binding of moisture, resulting in the reduction of water activity within the commodity (Naidoo and Lindsay, 2010). The reduction of a_w ultimately results in the inhibition of the microbial enzymes that are responsible for growth, degradation of food and general metabolism (Naidoo *et al*, 2010). Burnham *et al*, (2008) reported that *s.aureus* initial population is slightly decreased with several hurdle technologies including marination and dry cooking. Akkaya *et al*, 2014 determine the capability of *Staphylococcus aureus* to produce toxins in Turkish sucuk and results indicate that contamination is possible due to handling activity.

2.15.3. Survival of *S. aureus* in frozen storage temperature

Freezing is widely used to ensure the safety and quality of food. However, some research has shown that low temperatures are sublethal to *S. aureus*; at low temperatures, the *S. aureus* cell itself is damaged, but not dead (Suo *et al*,

2014). Moreover, when the contaminated frozen food is placed at suitable temperatures, the sublethally injured cells are resuscitated and can proliferate again; hence, *S. aureus* poses a severe threat to food safety (Haines, 1938; Archer, 2004; Wesche et al, 2009).

Sanjeev et al, (1986) determine the occurrence of coagulase-positive staphylococci in 66 samples of frozen fishery products consisting of frozen crab meat, frozen prawns, frozen cuttlefish and frozen mussel collected from nine cold stores situated in and around Cochin city (India). And found that 48 samples (72.72%) revealed the presence of coagulase-positive staphylococci.

Erkmen, 1997 found that the numbers of *S. aureus* cells were decreased by 2.02 log units in ground beef held at -27°C after 7 weeks. After 90 days storage at -20 °C, the numbers of *S. aureus* remained constant in the freezer (Metzger 2015). Normally, vegetative cells of Gram-positive bacteria are more resistant to environmental stresses such as freezing and low temperature (Silhavy et al, 2010).

Wu et al, (2014) reported that at an unfavorable storage temperature (-20± 2°C) 41.8% of the *S. aureus* organisms remained viable after 30 days storage and found, *S. aureus* can survive in precooked albacore and skipjack meat stored at -20 ± 2 °C for 4 wk with a less than 0.6 log CFU/g reduction of total cell counts.

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3. MATERIAL AND METHODS

3.1. Sample collection and preparation

Fresh Bombay duck (*Harpadon nehereus*), dry Bombay duck, Shrimp (*Parapenaeopsis styliifera*) and whiteleg shrimp (*Litopenaeus vannamei*) used in this study were collected from the Versova landing centre, Mumbai Maharashtra. Fresh samples collected from the landing centre were aseptically transported to the laboratory in chilled condition for bacteriological analysis and further cold storage experiments.

3.2. Work plan for storage study of samples

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolate was taken from a previous study (Kumar, 2015). For inoculation purpose two dilution concentration used (10^7 and 10^4 log CFU), to estimate the effect of different storage conditions in both low and high inoculated size in survival pattern of *S.aureus*. Surface inoculation of the MRSA culture was done into fresh and dried Bombay duck, peeled shrimp and cooked shrimp. These samples were stored at different temperature. Fresh Bombay duck was stored in ice, dry Bombay duck at room temperature, cooked and peeled shrimp at -20°C . The sample were analyzed for the survivability of MRSA by plate counting on Baird Parker Agar (BPA) at regular intervals.

3.3. Methicillin-resistant *S. aureus* (MRSA)

The isolate used for inoculations in this study was taken from a previous study by Kumar (2015) and was isolated from the salt used in fish fermentation. This strain harbors the efflux pump gene *lmrS* of *S. aureus*.

3.4. Methicillin-resistant *Staphylococcus aureus* reference culture

S. aureus ATCC BAA -976 was used as a positive control in this study in all experiments. This isolate is methicillin-resistant and also harbours the *lmrS* gene.

3.5. Media and ingredients

Bacteriological media used in this study were purchased from Hi-media Laboratories Pvt. Ltd. Mumbai. The composition and preparation of various media and ingredients used in this study are given in Appendix – 1. All the media were sterilised by autoclaving at 15 psi pressure at 121° C for 15 minutes.

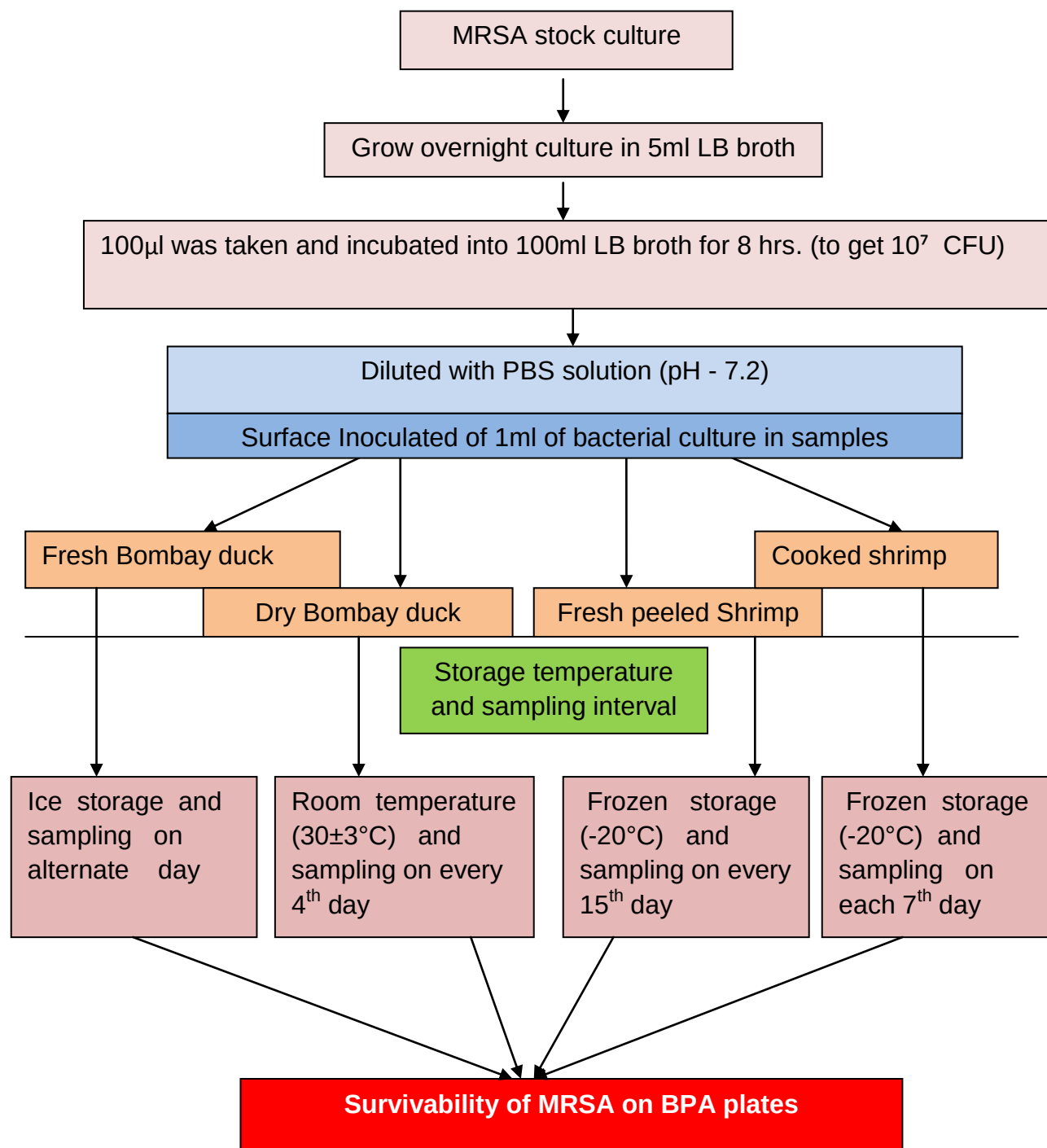
3.6. Preparation of glassware and petri plates

All of the glass wares and petri plates were soaked in the mild detergent solution for 5-10 minutes. Then they were washed thoroughly in tap water and rinsed with distilled water. After air-drying, all glass wares were sterilised in a hot air oven at 180°C for 2 hours, and plastic Petri plates were autoclaved at 121°C for 15 minutes. Microcentrifuge tubes, micro tips PCR tubes, etc. were sterilised by autoclaving at 121°C for 15 minutes.

Table 5. Description of samples

S.N o.	Sample selection site	Common name	Scientific name
1	Versova landing centre	Bombay duck (Bombil)	<i>Harpadon nehereus</i>
2	Versova fish market	Dried Bombay duck (sukhhat Bombil)	<i>Harpadon nehereus</i>
3	Versova landing center	Kiddi Shrimp	<i>Parapeneopsis stylifera</i>
4	Rohtak (Haryana)	White leg shrimp	<i>Litopenaeus vannamei</i>

3.7. Survivability study design for MRSA



3.8. Preparation of bacterial culture

Methicillin-resistant *Staphylococcus aureus* culture was subcultured from -80°C glycerol stock by inoculating into Luria Bertani broth (LB) and incubating at 37°C overnight. A loopful of the culture was transferred to 5 ml LB broth and incubated overnight at 37°C. From the overnight grown culture, 100 µl was transferred to 100 ml of nutrient broth and incubated for 8 hours at 37°C to produce a culture suspension of 10⁴ and 10⁷ log CFU/ml. The culture was diluted with phosphate buffer saline (PBS pH 7.2 Hi-media, Mumbai) to required levels for inoculation into the samples.

3.9. Preparation of fresh Bombay duck and shrimp for storage study

Fresh Bombay duck (*Harpadon nehereus*) and shrimp (*Parapeneopsis stylifera*) samples were collected from the Versova landing centre, Mumbai. The samples were packed in sterile bags and transported to the laboratory in chilled condition. Fresh Bombay duck and shrimp were cut into uniform pieces of 10 g sizes, placed in a 50 ml graduated sterile tubes (Abdos, India) for storage studies. The samples were surface inoculated with 10⁷ or 10⁴ log CFU/g of methicillin-resistant *S. aureus* and stored at respective temperatures. MRSA counts were determined by enumerating *S. aureus* on Baird-Parker plates. The inoculated samples were held in a biological safety cabinet for about 5 minutes to allow the absorption of the inoculums onto the samples. Uninoculated samples were used as controls.

Fresh Bombay duck



Fresh Shrimp



Cooked Shrimp



Dry Bombay duck



3.10. Preparation of cooked shrimp for storage study

Litopenaeus vannamei was collected from Rohtak (Haryana) in frozen condition. The sample was thawed in running water for 30 minutes and preprocessing was done under hygienic conditions. The shrimp was cooked under steam for 15 minutes and cut into uniform pieces of 10 g sizes, placed in 50 ml graduated sterile tubes (Abdos, India). The samples were surface inoculated with MRSA culture and stored at -20°C.

3.11. Preparation of dry Bombay duck for inoculation study

Dry Bombay duck sample was collected from the Versova fish market, Mumbai and transported to the laboratory in a sterile bag. The sample was semi ground in a mixer grinder (Xpress Ruby plus mixer KM520 India), and 10-gram portions were placed in 50 ml graduated sterile tubes (Abdos, India) for the storage study at normal room temperature (30±3°C).

Table 6. Storage temperature for different types of samples

S.No.	Name of the sample	Storage condition
1	Fresh Bombay duck	In ice
2	Fresh shrimp	Frozen (-20°C)
3	Cooked shrimp	Frozen (-20°C)
4	Dry Bombay duck	Room temperature (30±3°C)

3.12. Microbial analysis and confirmation of MRSA in fresh and dry Bombay duck and peeled and cooked shrimp

Inoculated samples were analysed for total aerobic plate count, and MRSA counts immediately after inoculation (0-day count). Stored fresh Bombay duck was sampled on alternate days, frozen peeled shrimp at 15- day, cooked shrimp at 7- day intervals, and dry Bombay duck at 4 - day intervals.

3.12.1. Enumeration, isolation and identification of MRSA from the inoculated samples

Ten-grams of the inoculated sample was homogenised with 90 ml phosphate buffered saline (PBS, pH 7.2) in a laboratory stomacher and the homogenate was 10-fold serially diluted in PBS (1:10). From each dilution, 0.1 ml aliquots were spread in duplicates on Baird Parker Agar (BPA) supplemented with 3.5% egg yolk tellurite emulsion. The plates were incubated at 37°C for 24 – 28 h. Typical colonies of *S. aureus* on BPA appear as circular, smooth, convex, moist 2-3 mm in diameter, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and a clear outer zone. and having buttery to gummy consistency when touched with inoculating needle (FDA, 2001). The typical colonies were picked from BPA plates and transferred to Luria Bertani agar plates for further confirmation by PCR.

3.12.2. Determination of Total Plate Count (TPC) of samples

Ten-grams the sample was mixed with 90 ml phosphate buffered saline (PBS) and homogenised in a laboratory stomacher. The homogenate was 10-fold serially diluted in PBS Phosphate buffer, and 0.1 ml of the dilutions were spread plated on duplicates on plate count agar for enumeration of total bacteria in samples. The plates were allowed to dry in an upright position and incubate at 37°C for 24 – 28 h (FDA 2001).

3.12.3. Moisture estimation in dry Bombay duck (A.O.A.C.2005)

The moisture content of the sample was analysed by the hot air oven method. About 10g of the sample was weighed in a petri dish which one previously dried in an oven. Distribute the sample evenly in the dish. Place the dish in a hot air oven maintained at 100°C ± 2° C to dry for 16- 18 hrs after that the dish was taken from the oven and cooled in a desiccator to room temperature. Final weight was taken by the dish with the sample.

$$\text{Moisture (\%, wet weight)} = \frac{W_1 - W_2}{W_1 - W} \times 100$$

Where

W = Weight in grams of empty petri dish

W1 = Weight in grams of the dish with the material before drying

W2 = Weight in grams of the dish with the sample after drying

3.13. Confirmation of MRSA using DNA based methods

3.13.1. Preparation of DNA

The DNA for PCR was prepared by crude lysate method. In a sterile 1.5ml centrifuge tube, 200 µl of 1XTE (10 mM Tris, pH 8.0; 1Mm EDTA) buffer was taken in which a loopful of the colony from an 18 - 24 hrs old culture on Luria Bertani agar plates was suspended and uniformly mixed using a vortex mixer. The bacterial cells in the suspension were lysed by placing the tube in a dry bath maintained at 98 °C for 15 minutes followed by immediate chilling on ice for rapid release of DNA. The cell debris was pelleted by centrifuging at 10,000 rpm for 2 minutes. The clear supernatant containing DNA was stored at -20°C for further use.

3.13.2. Polymerase Chain Reaction (PCR)

In a sterile 0.2 ml PCR tube or microcentrifuge tube, the following ingredients were mixed in the following reaction.

Table 7. Composition of PCR reaction

PCR master mix	Amount (μL)
Sterile milliQ water	18.2
10X Standard <i>Taq</i> Buffer	3
(dNTP) mix (2.5 mM)	0.5
Forward primer (10 picomoles/μl)	3
Reverse primer (10 picomoles /μl)	3
<i>Taq</i> DNA polymerase(5 U /μl)	0.3
Template DNA	2
Total volume	30

PCR was performed in a final reaction volume of 30 μl containing mixture and respective template DNA. Oligonucleotide primers specific for *Staphylococcus aureus* thermonuclease (*nucA*) gene (Brakstad *et al*, 1992), lincomycin resistant –gene *S. aureus* (*lmrs*) were used for PCR amplification. The primer sequences and amplification conditions are given in the Table 8. Amplification was done in a thermal cycler (Hi-media, Mumbai, India). DNA extracted from the reference strain ATCC BAA-976 (*S. aureus*) was used as positive control in all PCR assays.

Table 8. Nucleotide sequence for different primers

Primer	Target gene	Nucleotide sequence(5' - 3')	Products size
nuc	<i>nucA</i>	FP- GCGATTGATGGTGATACGGTT RP-AGCCAAGCCTTGACGAACTAAAGC	267
LmrS1	<i>lmrS</i>	FP- TAACAACCCGGAGACGAAAC RP- CCCATCGCCGCTAAGTATAA	241
Lmrs2	<i>lmrS</i>	FP- TGGGTTCGGGTTAATGCTATAC RP- AGTCCTGATAACAAGGCAGATAAA	287

Table 9. Thermocycling conditions used in this study

Primers	Initial denaturation	Denaturation	Annealing	Extension	Final extension
LmrS1	94°C-5 min	94°- 1 min	55°C- 1 min	72°C- 1 min	72°C- 2 min
Lmrs2	94°C-5 min	94°C - 1 min	55°C- 30 sec	72°C- 1 min	72°C- 2 min
nuc	94°C- 5 min	94°C - 1 min	55°C-30 sec	72°C- 1 min, 30 sec	72°- 3 min, 30 second

3.13.3. Analysis of PCR products

The amplification products were analysed were by agarose electrophoresis in 1XTAE buffer (pH 8.0). Agarose gels (1.5%) containing ethidium bromide (0.5 µg/ml) were prepared, and the PCR products were loaded after mixing with 6X loading dye. The electrophoresis was performed at 100 volts for 20-30 min.

The sizes of amplicons were determined by comparing with a reference DNA ladder (GeneRuler, Thermo Fisher Scientific, USA). The gels were visualised and photographed using a gel documentation system (Bio-Rad, USA).

3.14. Study of *lmrS* gene by quantitative real time (RT) PCR

For all experiments, an overnight culture was prepared from a single MRSA colony in 5 ml LB broth and 100 µl of this was inoculated into 5 ml of fresh LB broth. Three LB broth tubes containing 5%, 10% and 15% salt (NaCl) were prepared and inoculated with the test strain. A control tube without salt was also inoculated. All tubes were incubated at 37°C for 8 h to get 10⁸ log CFU/ml. These sets were used to study the gene expressions at different salt concentrations.

To study the effect of temperature on *lmrS* gene expression, MRSA was grown in LB and the bacterial cells were pelleted by centrifugation. The pellets were resuspended in PBS and one set each was stored in ice and at -20°C.

Table 10. Primer sequences for 16SrRNA and *LmrS* gene

Primer	Target gene	Nucleotide sequence(5' - 3')	Product size
<i>Lmr</i>- RT1 and RT2	<i>lmrS</i>	FP-TGGTCGTGTAATGGCTGGTTCACT RP- AGCTGCCATAACAAGCACAGCTAC	180 bp
16S – 1 and 16S-2	16S rRNA	FP- CGGTCCAGACTCCTACGGGAGGCAGCA RP- GCGTGGACTACCAGGGTATCTAACC	455 bp

3.14.1. Extraction of RNA and preparation of cDNA

The total RNA was extracted from all the samples using SV total RNA isolation kit (Promega, USA). One-ml of the culture was transferred into a 1.5 ml microcentrifuge tube and centrifuged for 2 minutes at 14,000 rpm at room temperature. Samples from -20°C and iced condition were centrifuge at 0°C as the previous sample. The supernatant was removed carefully and the pellet was resuspended in 100µl of freshly prepared TE containing lysozyme (3mg/ml) for 5 – 10 minutes. Then 75 µl of RNA lysis buffer was added along with the addition of 350 µl of RNA dilution buffer and mixed by inversion. Into this, 200 µl of 95% ethanol was added and mixed by pipetting 3 – 4 times. This lysate was transferred to spin column assembly and centrifuge at 12,000rpm for 1 minute. The liquid was discarded from the collection tube, and 600 µl of RNA wash solution was added and centrifuged at 12,000rpm for 1 minute. An incubation mixture was prepared with 40 µl Yellow Core Buffer, 5 µl 0.09M MnCl₂ and 5 µl of DNase I enzyme for each sample. From the mixture, 50µl was directly applied to the membrane inside the spin basket and incubated at room temperature for 15 minutes to remove genomic DNA. After the incubation, 200µl of DNase stop solution was added and centrifuged at 12,000rpm for 1 minute. The column was washed with 600µl of RNA wash solution and centrifuged at 12,000 rpm for 1 minute. A second washing was done with 250 µl of RNA wash solution. The spin basket was transferred to the capped 1.5 ml elution tube, and 100µl nuclease-free water was added to the membrane. RNA was eluted by centrifuging at 12,000 rpm for 1 minute.

3.14.2. First strand cDNA synthesis

Extracted RNA was converted into cDNA using GoScript™ Reverse transcription system (Promega, USA) according to manufacturer's instructions (Table 11). RNA (3 µl) was added along with random primer to a total volume of 5µl. The mixture was incubated at 70°C in the dry bath for 5 minutes and kept on ice until reverse transcription mixture was added.

Table 11. Composition of cDNA synthesis mixture

COMPONENT	VOLUME(μl)
Experimental RNA (up to 5 μ g/reaction)	3
Primer [Oligo(dT)15 (0.5 μ g/reaction) and/or Random Primer (0.5 μ g/reaction) or gene-specific primer (10–20pmol/reaction)]	1
Nuclease-Free Water	1
Total volume	5

3.14.3. Preparation of reverse transcription mixture**Table 12. Composition of reverse transcription reaction mixture**

COMPONENT	VOLUME (μl)
GoScript™ 5X Reaction Buffer	4
MgCl ₂ (final concentration 1.5–5.0mM) ¹	1.5
PCR Nucleotide Mix (final concentration 0.5mM each dNTP) ²	1
Recombinant RNasin® Ribonuclease Inhibitor (optional)	0.5
GoScript™ Reverse Transcriptase	1
Nuclease-Free Water (to a final volume of 15 μ l)	7
Reverse transcript mix	5
Total volume	20

Reverse transcription was performed with the following conditions; 25°C for 5 minutes, followed by 42°C for 1 h. Inactivation of reverse transcriptase was done by incubating at 70°C for 15 minutes.

3.15. Real-time PCR for extracted cDNA samples

Table 13. Real-time PCR reaction for extracted cDNA samples

RT-PCR mixture	Amount (µl)
Master mix	10
Forward primer	2
Reverse primer	2
Water	4
cDNA	2
Total volume	20

Table 14. Thermocycling conditions used in this study

Primer	Initial denaturation	Denaturation	Annealing	Extension
16S -RNA	95°C for 3 min.	95°C for 20 sec.	55°C for 30 sec.	72° for 30 sec.
RT-Lmr	95°C for 3 min	95°C for 20 sec.	55°C for 30 sec.	72° for 30 sec

4. RESULTS

4.1. Determination of the growth curve of *Staphylococcus aureus*

The growth of *Staphylococcus aureus* in Luria Bertani (LB) broth was monitored spectrophotometrically at 660 nm and a growth curve was determined. The readings were taken at 2 h intervals for 16 h. The growth curve showed that *S. aureus* reached exponential phase in 8 h and remained in stationary phase up to 28 h.

Table 15. Growth curve of methicillin resistant *Staphylococcus aureus*

Time (in hrs.)	Absorbance (OD _{660nm})
0	0
2	0.1090
4	0.2495
8	0.4734
12	0.5080
16	0.5219
28	0.5405

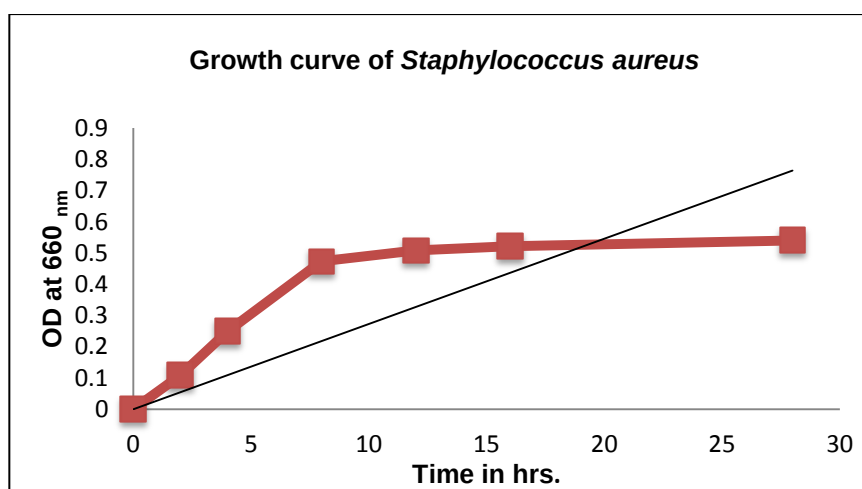


Fig. 1. Growth curve of methicillin resistant *Staphylococcus aureus*

4.2. Survivability of methicillin resistant *S. aureus* (MRSA) in fish and fishery products

Fresh Bombay duck, dried Bombay duck and fresh shrimp were inoculated with *S. aureus* and the survivability of the bacterium was studied. Fresh Bombay duck was stored in ice, dried Bombay duck at room temperature, and fresh and cooked shrimp at -20°C. *S. aureus* was inoculated at a higher contamination level of 10^7 (7 log) CFU and a medium contamination level of 10^4 (4 log) CFU into all the samples separately.

4.2.1. Survivability of MRSA in Bombay duck at higher inoculation level (10^7 CFU) during ice storage

Fresh Bombay duck inoculated with 10^7 CFU. *S. aureus* was stored in ice for up to 18 days and the microbiological analysis was done on alternate days. The fresh sample was tested for the presence of MRSA and it was found to be free from MRSA. The samples were analyzed for the total plate count (TPC) during the storage period. Immediately after inoculation (0 h), the sample had an initial MRSA count of 6.45 log CFU/g (Table 16). On day-10, the count suddenly decreased to 3.516 log CFU/g. At the end of storage period (day-18), a MRSA count of 3.702 Log CFU/g. was recorded (Fig. 6).

Table 16. Survivability of MRSA in Bombay duck stored in ice and inoculated with 10^7 CFU

Time (days)	log CFU/g
0	6.454 ^a ±0.0032
2	6.402 ^a ±0.0133
4	5.405 ^b ±0.0036
6	5.018 ^c ±0.0146
8	5.022 ^c ±0.3203
10	3.516^f±0.2194
12	5.342 ^b ±0.0139
14	5.328 ^b ±0.0028
16	4.047 ^d ±0.0137
18	3.702^e±0.0300

Values reported as mean ± standard deviation (n=2)

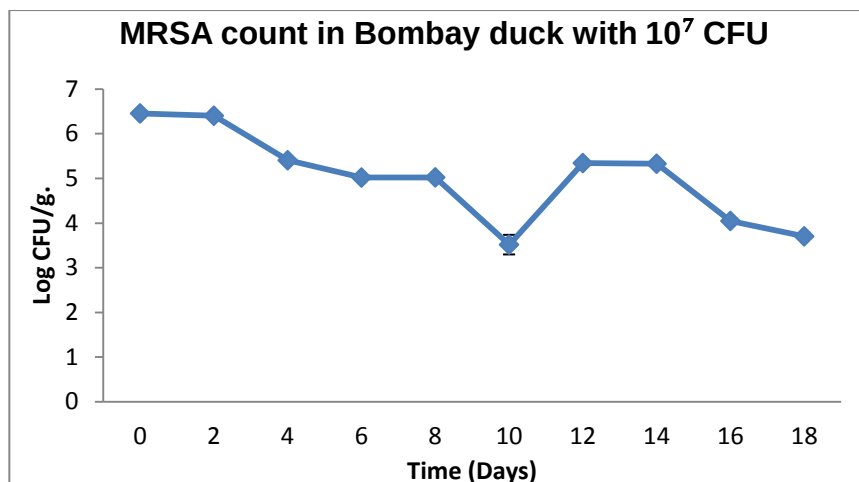


Fig.2. MRSA count in Bombay duck inoculated with 10⁷ CFU

4.2.2. Survivability of MRSA in Bombay duck at lower inoculation level (10⁴ CFU) during ice storage

Bombay duck was inoculated with lower level (10⁴ CFU) of MRSA and stored in ice. As above, sampling was done on alternate days, starting from day-0 (Table 17). The initial (0-day) count was 3.296 log CFU/g. However during ice storage, the MRSA count gradually increased up to 5.362 log CFU/g and from day-6, the counts started declining (Fig. 6). The final count on day-15 was 3.010 log CFU/g

Table 17. Survivability of MRSA in Bombay duck stored in ice and inoculated with 10⁴ CFU

Time(Days)	Log CFU/g.
0	3.296 ^d ±0.0928
2	4.064 ^b ±0.0052
4	4.075 ^b ±0.0206
6	5.362^a±0.0372
8	4.077 ^b ±0.0128
10	3.600 ^c ±0.0461
12	3.951 ^b ±0.0240
14	3.459 ^d ±0.1370
16	3.379 ^d ±0.0256
18	3.010^e±0.2340

Values reported as mean ± standard deviation (n=2)

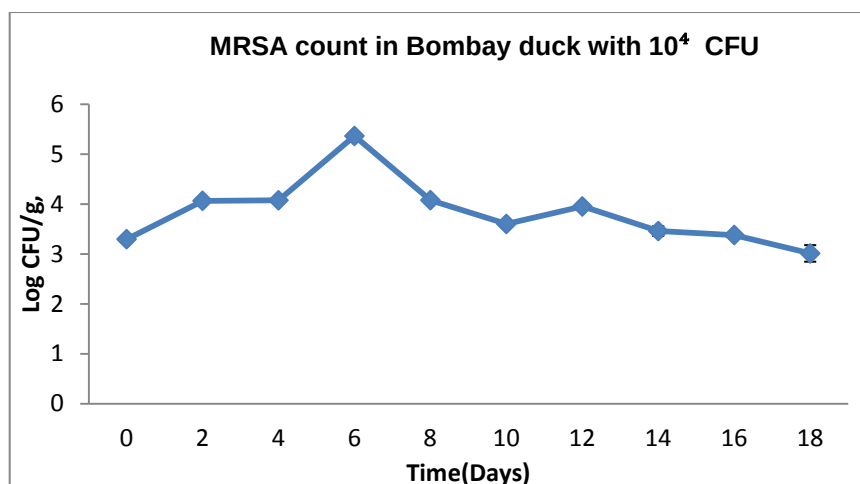


Fig. 3. MRSA count in Bombay duck inoculated with 10⁴ CFU

4.2.3. Total plate count (TPC) count in fresh Bombay duck during ice storage

The fresh Bombay duck was analyzed for total plate count (TPC) count. The initial (0-day) bacterial count was 4.304 log CFU/g. (Table 18). Unlike MRSA, the total bacterial count increased and reached 7.325 log CFU/g on day-16 (Fig. 8).

Table 18. Total plate count (TPC) count in fresh Bombay duck during ice storage

Time (Days)	Log CFU/g.
0	4.304 ^a ±0.0106
2	4.348 ^b ±0.0082
4	5.356 ^c ±0.0202
6	5.378 ^c ±0.0025
8	6.384 ^d ±0.0088
10	6.393 ^d ±0.0012
12	6.397 ^d ±0.0049
14	6.407 ^d ±0.0216
16	7.325 ^e ±0.0101

Values reported as mean ± standard deviation (n=2)

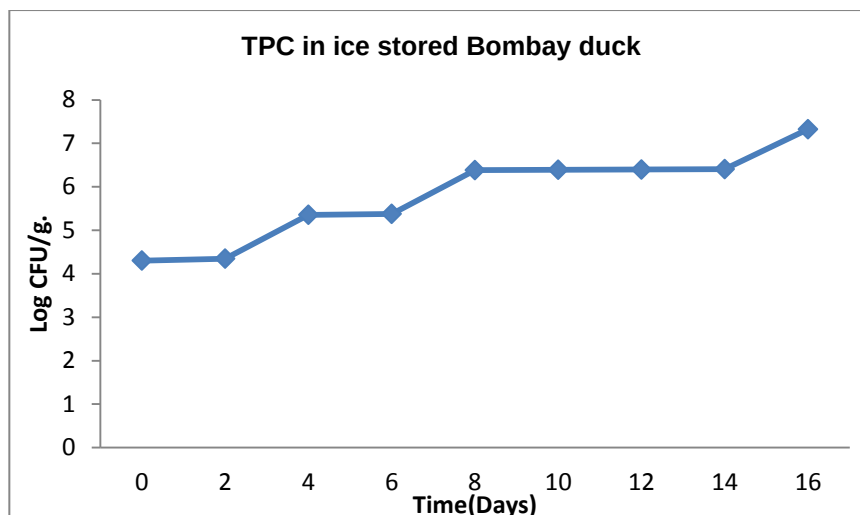


Fig. 4. TPC in ice stored Bombay duck

4.3. Estimation of moisture content in dry Bombay duck by direct heating method

The moisture from the dry Bombay duck was estimated in triplicates of the same sample.

Table 19. Moisture estimation in dry Bombay duck by direct heating method

Observation	Sample 1	Sample 2	Sample 3
Weight in grams of empty petri dish	36. 60	37.05	31.65
Weight in grams of the dish with the material before drying W1	45. 88	46.24	41.62
Weight in grams of the dish with the sample after drying W2	44.56	44.94	40.24

$$\text{Moisture (\%, wet weight)} = \frac{W1 - W2}{W1 - W} \times 100$$

I. 14.21%

II. 14.13%

III. 13.85%

So the average Moisture content in dry Bombay duck was about

$$\frac{14.21+14.13+13.85}{3} = 14.06 \%$$

4.3.1. Survivability of MRSA in dried Bombay duck stored at room temperature

Dried Bombay duck was inoculated with 10^7 and 10^4 CFU MRSA and stored at room temperature. MRSA counts and the total plate counts were determined at 4 days intervals for upto 20 days.

4.3.2. Survivability of MRSA in dry fish at higher inoculation level (10^7 CFU) and stored at room temperature and

The initial level of MRSA (0-day) immediately after inoculation was 5.42 log CFU/g which continuously decreased during storage period and reached 2.15 log CFU/g on day-20 of storage. The data is shown in Table 20.

Table 20. Survivability of MRSA in dry fish stored at room temperature and inoculated with 10^7 log CFU MRSA

Time(Days)	Log CFU/ g.
0	5.424 ^a ±0.0231
4	5.246 ^a ±0.0052
8	4.151 ^b ±0.0820
12	3.764 ^c ±0.0685
16	3.330 ^d ±0.1420
20	2.150^e±0.2128

Values reported as mean ± standard deviation (n=2)

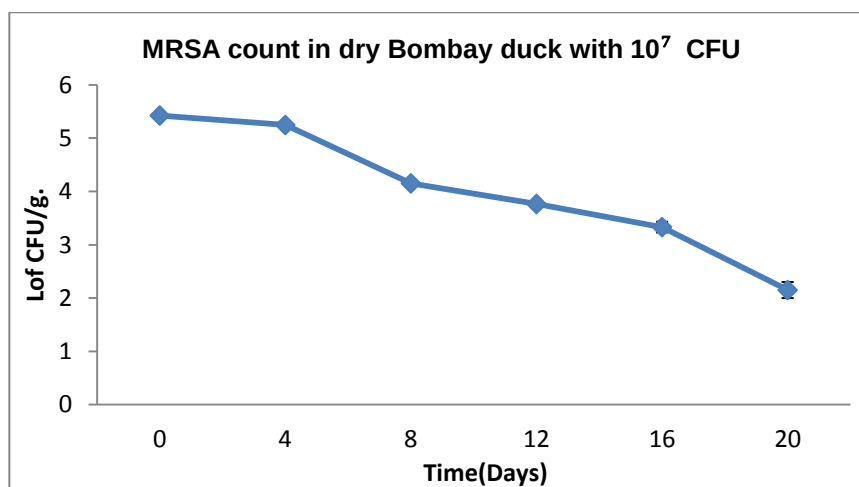


Fig. 5. MRSA count in dry Bombay duck inoculated with 10^7 CFU

4.3.3. Survivability of MRSA in dry fish at lower inoculation level (10^4 CFU) and stored at room temperature

Initial MRSA count immediately after inoculation with 10^4 log CFU was 3.56 log CFU/g on 0-day. During storage of at room temperature, the count continuously decreases and reached 2.66 log CFU/g (Table 21, Fig. 10).

Table 21. Survivability of MRSA in dry fish stored at room temperature and inoculated with 10^4 log CFU MRSA

Time (Days)	Log CFU/g.
0	3.567 ^b ±0.1480
4	5.040 ^a ±0.0335
8	4.778 ^a ±0.0765
12	3.346 ^b ±0.0963
16	3.190 ^c ±0.1568
20	2.661 ^d ±0.2601

Values reported as mean ± standard deviation (n=2)

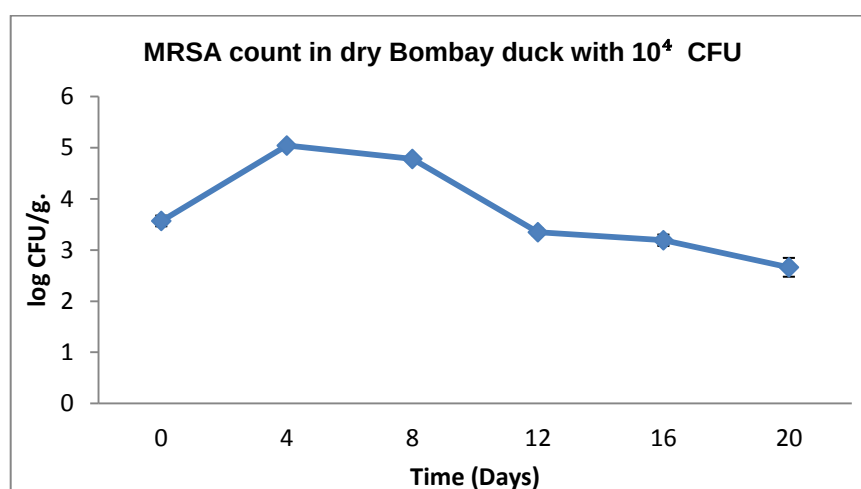


Fig. 6. MRSA count in dry Bombay duck inoculated with 10^4 CFU

4.3.4. Total plate count (TPC) in dry Bombay duck stored at room temperature

The sample was initially analyzed for total aerobic plate count, which was found to be 4.115 log CFU/g. (day-0). Subsequently during storage, the count increased by about 1.283 log CFU/g in the first four days reaching 5.394 log CFU/g. After this initial increase, the TPC continuously decreased and reached 3.19 log CFU/g. The data is shown in the Table 22.

Table 22. Total plate count (TPC) in dry Bombay duck stored at room temperature

Time (Days)	Log CFU/g.
0	4.115 ^c ±0.0259
4	5.394 ^a ±0.0198
8	5.280 ^b ±0.0160
12	4.116 ^c ±0.0469
16	3.507 ^d ±0.0855
20	3.190^e±0.0198

Values reported as mean ± standard deviation (n=2)

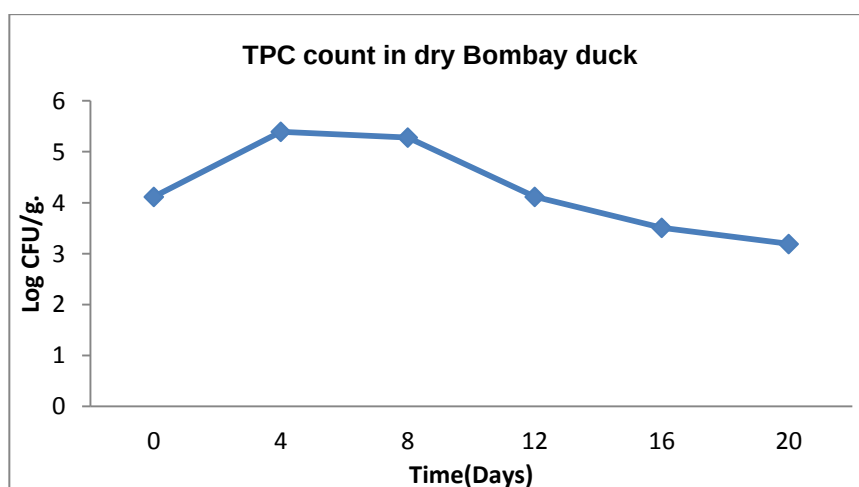


Fig. 7. TPC in dry Bombay duck

4.4. Survivability of MRSA in peeled shrimp stored at -20°C

Peeled shrimp (*Parapenaeopsis styliifera*) was inoculated with two levels (10^7 and 10^4) of MRSA and stored at -20°C. MRSA and total plate counts were taken at 15 days intervals.

4.4.1. Survivability of MRSA in peeled shrimp at higher inoculation level (10^7 CFU) during frozen storage

Peeled Shrimp (*Parapenaeopsis styliifera*) was inoculated with 10^7 and 10^4 CFU of MRSA separately and stored at -20°C. The initial (0-day) MRSA count in shrimp inoculated with 10^7 CFU was 5.443 log CFU/g. The count decreased during storage by about 1.524 log CFU/g and reached 3.919 log CFU/g on day-60 (Table 23 and Fig. 12).

Table 23. Survivability of MRSA in peeled shrimp stored at -20°C and inoculated with 10^7 log CFU

Time (Days)	Log CFU/g.
0	5.443 ^a ±0.0243
15	5.373 ^a ±0.0350
30	5.328 ^a ±0.0201
45	4.299 ^b ±0.0107
60	3.919^c±0.0883

Values reported as mean ± standard deviation (n=2)

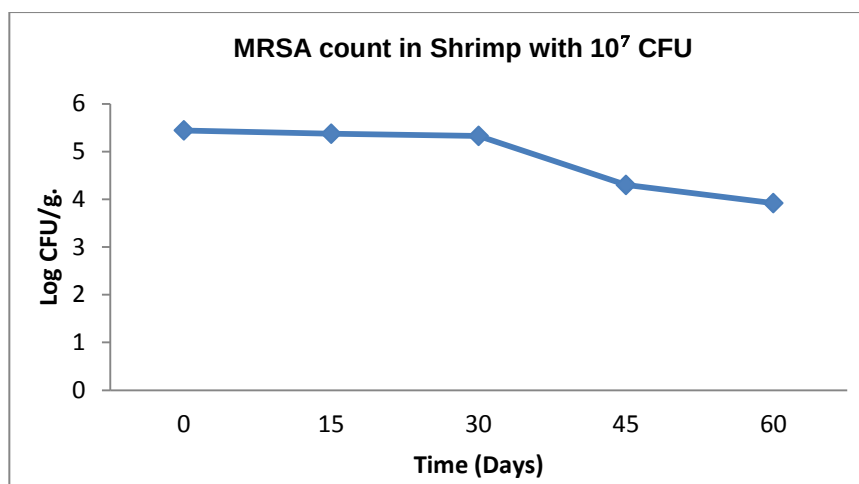


Fig. 8. MRSA count in peeled shrimp inoculated with 10^7 CFU

4.4.2. Survivability of MRSA in peeled shrimp at lower inoculation level (10^4 CFU) during frozen storage

The initial (0-day) MRSA count in the inoculated sample was 3.507 log CFU/g. During storage, the MRSA count remained more or less stable, although there was an increase of 0.8 log CFU/g till day-30 which subsequently started decreasing (Table 24). The final count on day-60 was 3.736 log CFU/g (Fig. 13).

Table 24. Survivability of MRSA in peeled shrimp stored at -20°C and inoculated with 10^4 log CFU

Time (Days)	Log CFU/g.
0	$3.507^b \pm 0.2062$
15	$4.351^a \pm 0.0410$
30	$4.199^a \pm 0.0135$
45	$3.754^b \pm 0.0540$
60	$3.736^b \pm 0.0785$

Values reported as mean $\pm$ standard deviation (n=2)

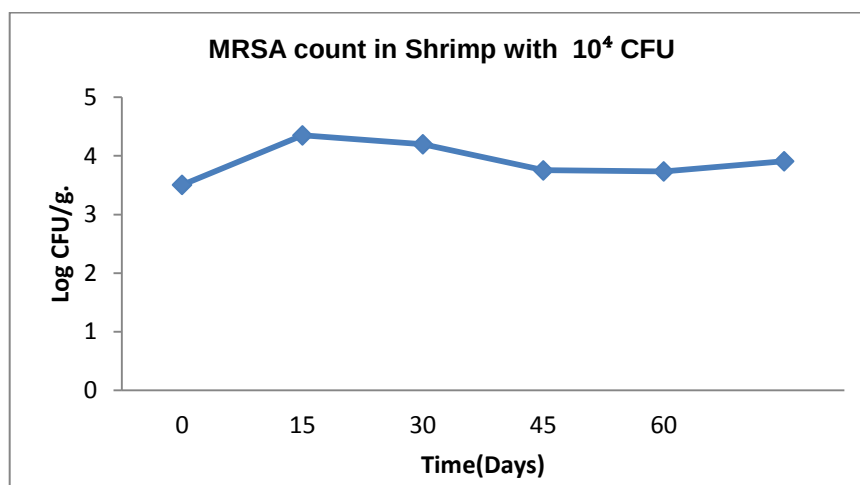


Fig. 9. MRSA count in peeled shrimp inoculated with 10⁴ CFU

4.4.3. Total plate count (TPC) in peeled shrimp stored at -20°C

The fresh sample after peeling was analyzed for the total plate count, and it was found that the initial count was 5.11 log CFU/g. The count decreased by 1.85 log CFU/g and finally reached 3.26 log CFU/g on day-60. The data is shown in the Table 25.

Table 25. Total plate count (TPC) in peeled shrimp stored at -20°C

Time (Days)	Log CFU/g.
0	5.118 ^a ±0.0210
15	4.933 ^a ±0.0821
30	3.896 ^b ±0.0821
45	3.896 ^b ±0.0467
60	3.268 ^c ±0.1312

Values reported as mean ± standard deviation (n=2)

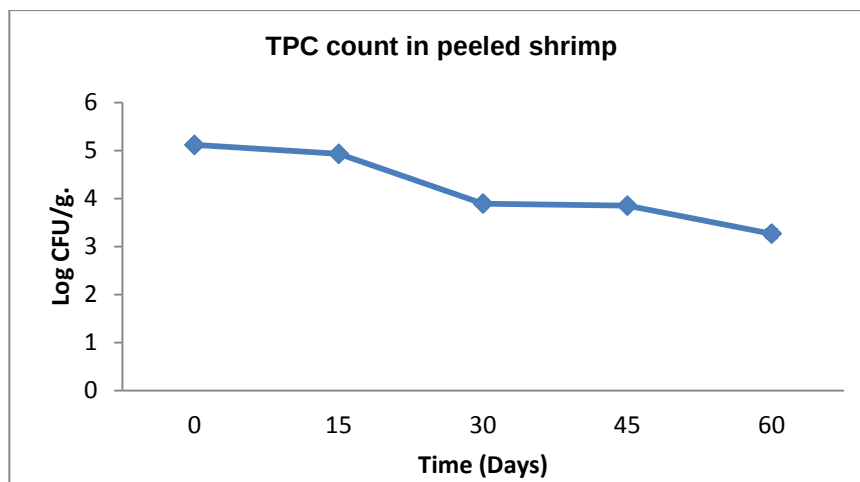


Fig.10. TPC in peeled shrimp

4.5. Survivability of MRSA in cooked shrimp stored at -20°C

For cooked shrimp storage study *Litopenaeus vannamei* is used as a sample. The sample was inoculated with 10^7 CFU MRSA and stored at -20°C. The counts were determined at weekly intervals for up to 28 days.

4.5.1. Survivability of MRSA in cooked shrimp at higher inoculation level (10^7 CFU) during frozen storage

The initial (0-day) MRSA count was 5.317 CFU/g (Table 26) which remained unchanged till day-14. This was followed by a small but gradual decline in the MRSA count reaching 3.81 CFU/g on day-35. The data is shown in Table 26 and depicted in Fig. 15.

Table 26. Survivability of MRSA in cooked shrimp stored at -20°C and inoculated with 10^7 log CFU

Time (Days)	log CFU/g
0	5.317 ^a ±0.0044
7	5.279 ^a ±0.0112
14	5.185 ^a ±0.0380
21	4.361 ^b ±0.0267
28	3.886 ^c ±0.1228

Values reported as mean ± standard deviation (n=2)

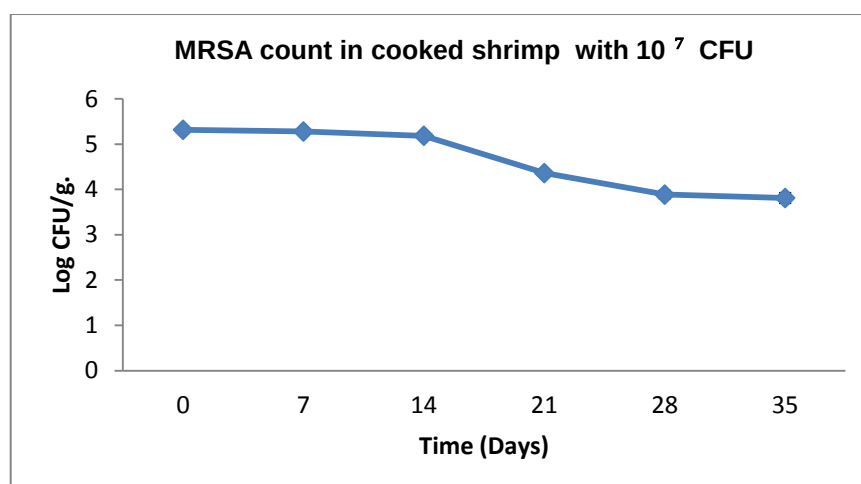


Fig. 11. MRSA count in cooked shrimp inoculated with 10^7 CFU

4.5.2. Total plate count (TPC) in cooked shrimp stored at -20°C

Cooked shrimp was analyzed for total plate count starting from day-0, i.e., immediately after inoculation. The initial TPC was low, 3.631 log CFU/g. The count increased up to day-7 and gradually declined to 4.045 log CFU/g. by day-21 and the count finally reached 3.268 log CFU/g. on day-35 (Table 27 and Fig. 16).

Table 27. Total plate count (TPC) in cooked shrimp stored at -20°C

Time (Days)	Log CFU/g.
0	3.631 ^c ±0.0572
7	4.322 ^a ±0.0087
14	4.279 ^a ±0.0209
21	4.045 ^b ±0.0166
28	3.916 ^b ±0.0260
35	3.268^d±0.1312

Values reported as mean ± standard deviation (n=2)

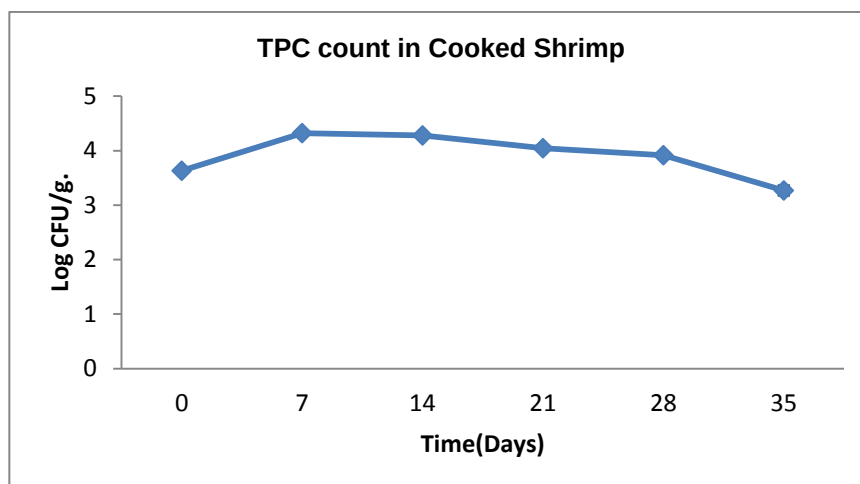


Fig. 12. TPC of cooked shrimp

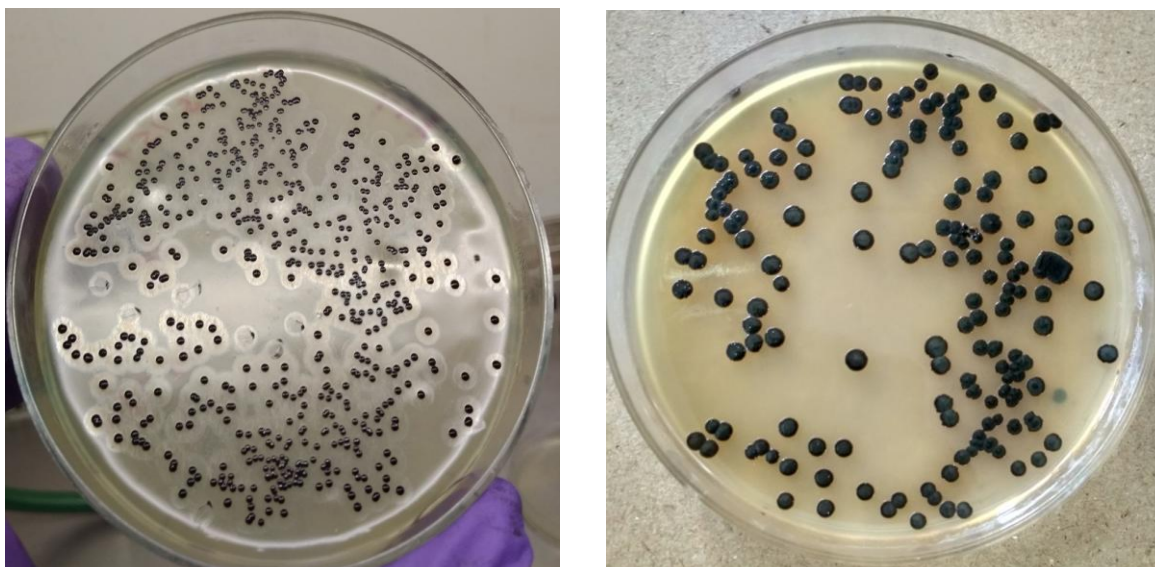


Fig. 13. Typical colonies of Methicillin resistant *Staphylococcus aureus* and *Micrococcus caseolyticus* on BPA Plates



Fig. 14. Methicillin resistant test for MRSA strain

4.6. Molecular characterization of methicillin resistant *Staphylococcus aureus*

4.6.1. PCR detection of *lmrS* gene using LMRS-1 primers

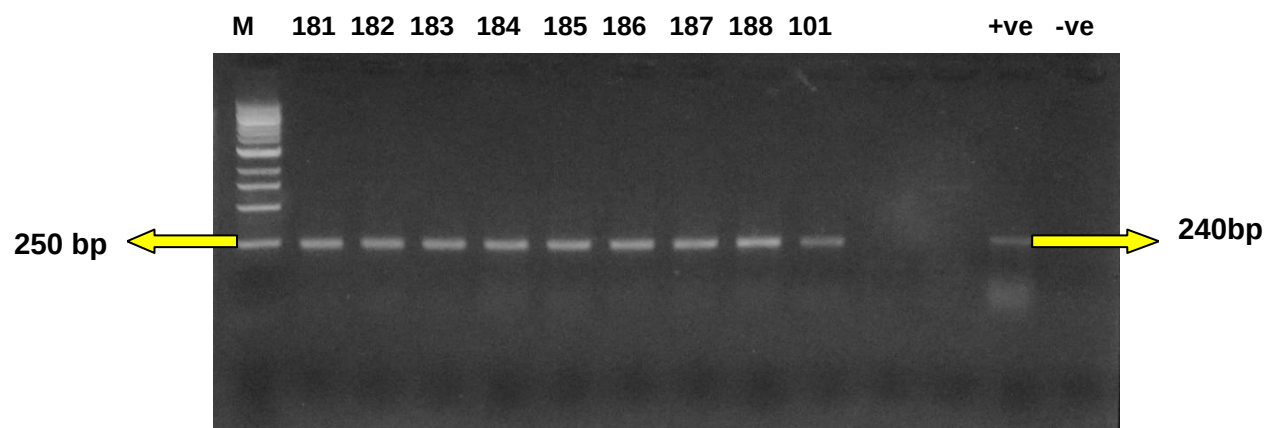


Fig. 15. PCR detection of *lmrS* gene using Lmrs-1 primers

Lane M	GENESTA™ 1 Kb DNA ladder
Lane 181 - 184	Isolate from shrimp inoculated with 7 log CFU
Lane 185 – 188	Isolate from Shrimp inoculated with 4 log CFU
Lane 101	Isolate from Bombay duck inoculated with 7 log CFU
+ve	MRSA positive control
-ve	Negative control

4.6.2. PCR detection of *lmrS* gene using LmrS-2 primers

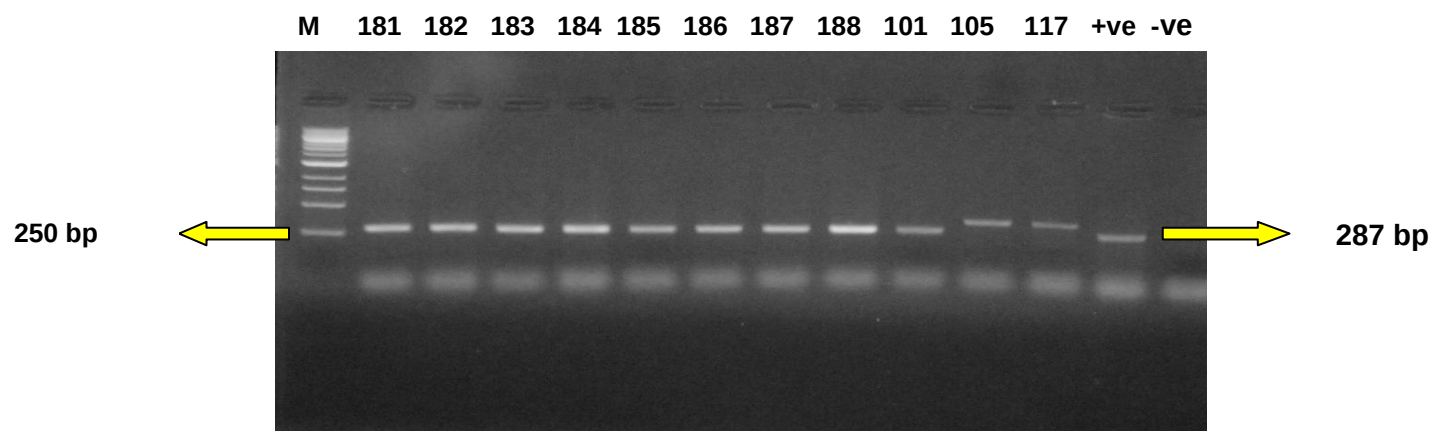


Fig. 16. PCR detection of *lmrS* gene using LmrS-2 primers

Lane 181-184	Isolate from shrimp inoculated with 7 log CFU
Lane 185 - 188	Isolate from Shrimp of inoculated with 4 log CFU
Lane 101	Isolate from Bombay duck inoculated with 7 log CFU
Lane 105	Isolate from Bombay duck at 12 th day
Lane 117	Isolate from dry Bombay duck at 12 th day
+ve	MRSA positive control
-ve	Negative control

4.6.3. PCR detection of *nucA* gene of *S. aureus*

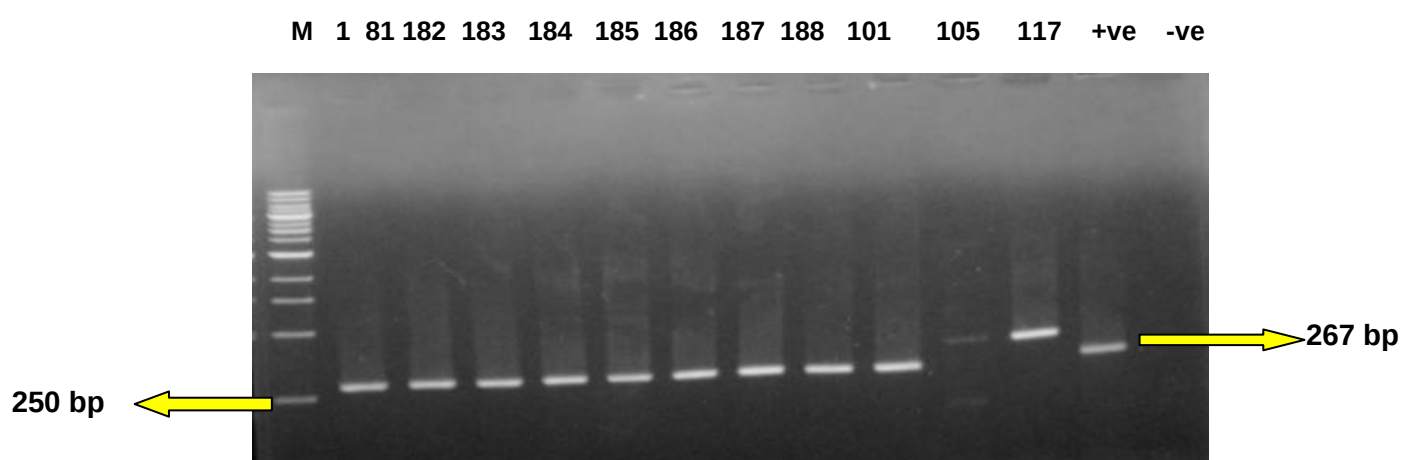


Fig. 17. Results of PCR assay for *nucA* gene

Lane M	GENESTA™ 1 Kb DNA ladder
Lane 181 – 184	Isolate from shrimp of 10 ⁷ log CFU/gm
Lane 186 – 188	Isolate from Shrimp of 10 ⁴ log CFU/gm
Lane 101	Isolate from Bombay duck of 10 ⁷ log CFU/gm
Lane 105	Isolate from Bombay duck at 12 th day
Lane 117	Isolate from dry Bombay duck at 12 th day
+ve	MRSA positive control
-ve	Negative control

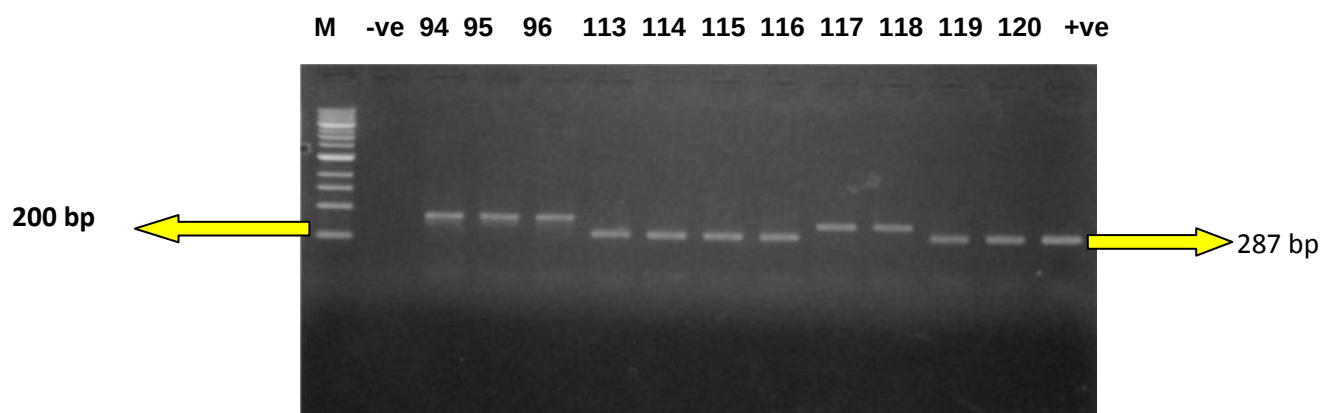


Fig. 18. PCR assay for isolates using Lmrs-2 primers

Lane M	Takara 1 Kb DNA ladder
Lane 94 – 96	Isolate from Bombay duck inoculated with 7 log CFU
Lane 113 – 116	Isolate from Dry Bombay duck inoculated with 7 log CFU
Lane 117-118	Isolate from Dry Bombay duck inoculated with 4 log CFU
Lane 119- 120	Isolate from Dry Bombay duck inoculated with 7 log CFU
+ve	MRSA positive control
-Ve	Negative control

Table 28. Sequencing results of *lmrS* PCR products

Isolate code	Sequence length	Max. identity	Organism
94	287	100 %	<i>Bacillus paralicheniformis</i>
95	287	100%	<i>Bacillus paralicheniformis</i>
96	287	99%	<i>Bacillus paralicheniformis</i>
101	287	99%	<i>Staphylococcus aureus</i>
105	287	98%	<i>Macrococcus caseolyticus</i>
117	287	97%	<i>Macrococcus caseolyticus</i>
125	287	99%	<i>Staphylococcus aureus</i>

Table 29. Sequencing results with *nucA* gene products

Isolate code	Sequence length	Max. identity	Organism
117	267	99%	<i>Macrococcus caseolyticus</i>
181	267	99%	<i>Staphylococcus aureus</i>
185	267	99%	<i>Staphylococcus aureus</i>
208	267	96%	<i>Macrococcus caseolyticus</i>

4.7. Extraction of RNA and preparation of cDNA from the MRSA after grown in different conditions

M RT 5% 10% 15% -20 Ice +Ve -Ve

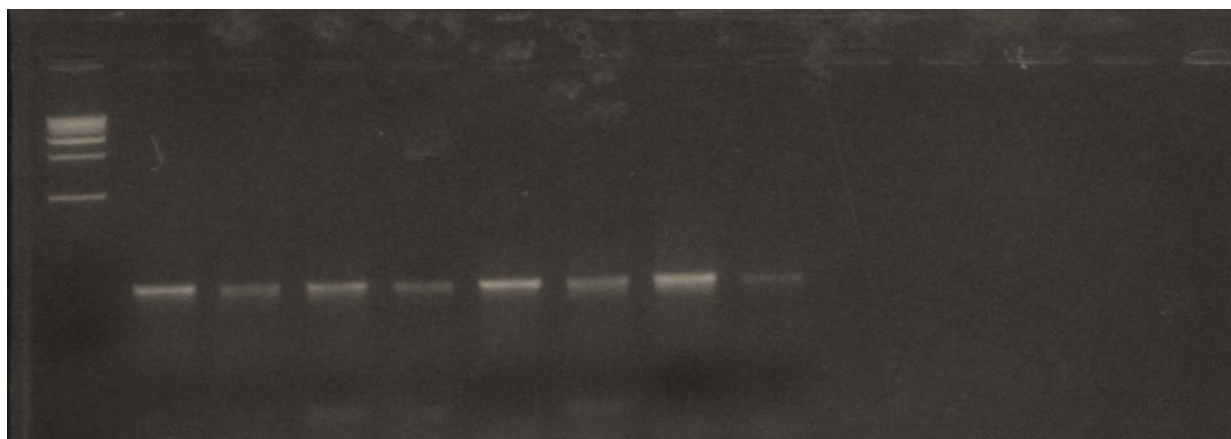


Fig. 19. PCR amplification of 16SrRNA gene from cDNA

Lane M	GENESTA™ 1 Kb DNA ladder
RT	Bacteria were grown at room temperature for 8 h.
5%, 10%, 15%	Bacteria were grown with different salt concentration for 8 h.
-20°C	Bacteria grown for 8 h and stored at -20°C for 4 hrs.
Ice	Bacteria grown for 8 h and stored in ice for 4 hrs.
+ve	MRSA positive control
-ve	Negative control

4.8. Expression of *Lmrs* gene in RT-PCR with different growth conditions

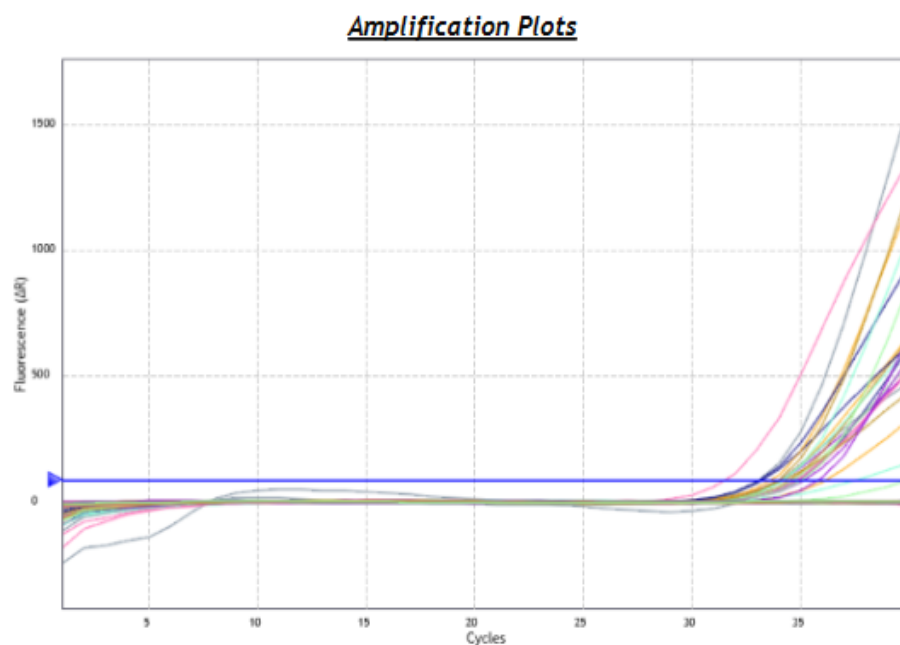


Fig. 20. Amplification of *Lmrs* gene by real-time (RT) PCR

Table 30. Cq (ΔR) values for MRSA grown in different environmental conditions

S.No.	Name of the sample	Cq (ΔR) values for 16S rRNA gene	Cq (ΔR) values for RT- <i>lmrS</i> gene
1	Bacteria grown for 8 h at normal room temperature (37°C)	35.7	34.305
2	Bacteria grown for 8 h in room temperature with 5% salt	36.365	34.845
3	Bacteria grown for 8 h in room temperature with 10% salt	34.255	33.58
4	Bacteria grown for 8 h in room temperature with 15% salt	34.755	33.055
5	Bacteria grown for 8 h in room temperature and kept in -20°C for 4 h.	33.885	35.99
6	Bacteria grown for 8 h in room temperature and kept in ice for 4 hrs.	34.69	34.08

5. DISCUSSION

S. aureus is a versatile pathogen which can grow in a wide range of salt, temperature, water activity and pH (Baeza 2007). Foods that are extensively handled during preparation and kept without refrigeration are usually associated with *Staphylococcal* food poisoning (Kereluk 1961). Humans and animals are natural carriers of *S. aureus* and the contamination of foods with *S. aureus* occurs due to unhygienic handling by food handlers. *S. aureus* does not compete well with other bacteria in foods and hence does not succeed in multiplying in foods when the background bacterial flora is high. Therefore, the presence of *S. aureus* and the consequent food-borne infections by this pathogen is associated with the foods that inhibit the growth of competing microorganisms such as frozen, canned salted, smoked fishery products, boiled fish paste and fish sausages (Bryan, 1980; Sanjeev *et al*, 1986; Nakano *et al*, 2004; Simon and Sanjeev, 2007).

Studies have reported the presence of *S. aureus* in salt used in fish processing and this is an important source of *S. aureus* in fish (Hansen *et al*, 1995). Kumar (2016) isolated MRSA from salt used in fish fermentation. The presence of *S. aureus* during the drying and smoking process of eels in Alaska has been reported (Eklund *et al*, 2004). In fresh fish, *S. aureus* may be introduced through hands of fish handlers, from containers, ice etc. The contamination is more serious when seafood such as shrimp are unhygienically handled during peeling before freezing in processing industries. Sanjeev *et al*, (1988) reported the presence of *S. aureus* in palms and throats of workers from fish processing factories in India. Ready-to-eat or ready-to-cook products have been shown to harbour *S. aureus* due to contamination during preparation and their multiplication during storage depending on the storage temperature. A study has shown that 3 out of 54 packages (5.5%) of vacuum-packed cold-smoked fish were contaminated with *S. aureus* (Gonzalez-Rodriguez *et al*, 2002).

According to the 4th Edition of the FDA (Food and Drug Administration of the United States) Fish and Fishery Products Hazards and Controls Guidance (FDA 2011), when significant manual handling occurs after cooking, there is a potential that fish and fishery products may become recontaminated with pathogens, including *S. aureus* (Xulei Wu and Yi-Cheng Su 2014). The problem of *S. aureus* contamination is more confounding when the bacterium becomes resistant to methicillin and other antibiotics. MRSA (methicillin-resistant *S. aureus*) is a significant human pathogen, although the role of foods in the transmission of this pathogen is not known. However, it is possible that *S. aureus* contaminating fish and fishery products may be MRSA and therefore their physiology of survival and persistence in fish and fishery products is of considerable interest.

In the present study, the survivability of methicillin-resistant *S. aureus* (MRSA) in fish and shrimp was studied. In case of fish, fresh and dried Bombay duck (*Harpadon nehereus*) were used separately for inoculation of *S. aureus*. The fresh Bombay duck was subjected to storage in ice, while the dried Bombay duck was stored at room temperature. In the case of shellfish, peeled shrimp (*Parapenaeopsis stylifera*) and cooked shrimp (*Litopenaeus vannamei*) were used, and these were frozen stored (-20°C) after inoculation with *S. aureus*. To represent high and low levels of contamination, *S. aureus* was inoculated at 10⁷ and 10⁴ CFU levels into all groups of samples. The study revealed that the survivability of MRSA in each sample type was different and was influenced by the storage temperature.

5.1 Survivability of MRSA in fresh Bombay duck stored in ice

Fresh Bombay duck was surface spiked with 10⁷ and 10⁴ CFU MRSA separately and stored in ice. Sampling for MRSA and total plate counts was done on alternate days. Tables 16, 17 and 18 show MRSA counts and total plate counts in the samples. In Bombay duck inoculated with 7 log CFU, the initial (day-0) count was 6.45 log CFU/g (Table 16). The MRSA counts gradually decreased in ice storage and reached 3.70 which corresponded to 42% decline in the population of *S. aureus* (Fig 2). From this point on, the counts of black colonies on BPA started increasing and

reached 5.32 log CFU/g on day-14 and again started declining. However, PCR analysis and microscopy revealed that the increase in the counts was due to the growth of *Bacillus paralicheniformis* and *Macrococcus caseolyticus*. The colonies of *M. caseolyticus* were dark black on BPA, but were larger than *S. aureus* and also lacked the halo around the colonies which characteristic of *S. aureus* colonies due to the lecithinase activity (Fig.13). The decrease in MRSA count might be due to the decrease in pH as a result of spoilage of fish and increase in the total bacterial number that *S. aureus* may not be able to compete with (Farrell and Upton 1978).

Akkaya *et al*, (2004) stated that the growth of *S. aureus* is affected by several factors including the population density of *S. aureus*, the presence and density of competitive microflora and the storage environment of the food, temperature, pH, salt, a_w and presence of O_2 . The observation that bacteria such as *M. caseolyticus* and *B. paralicheniformis* can dominate the BPA count in ice stored fish assumes significance. The roles of these bacteria in fish, their temperature tolerance and events leading to their predominance after a particular stage in ice storage need further investigation.

In fresh Bombay duck spiked with 4 log CFU of MRSA, the initial count was 3.29 log CFU/g on day-0. Interestingly, the total MRSA count increased by 2.07 log CFU/g and reached 5.36 log CFU/g on day-6 (Table 17). From day-8, the MRSA counts showed decreasing trend and reached 3.70 log CFU at the end of the storage period (day-18) (Fig.3). The results suggest that the behaviour of MRSA in ice-stored fish is different when present in different population densities. When MRSA population is in the range of 3 or 4 log CFU, the bacterium is able to adapt well, obtain nutrients and survive in the chilled condition in Bombay duck. Apart from the observed population dynamics of MRSA, complex interactions among *S. aureus* and between *S. aureus* and other bacteria in fish may exist.

The initial total plate count (TPC) of freshly Bombay duck was about 4.0 log CFU/g (Table 18) and during storage in ice, the counts continuously increased and reached 7.32 log CFU/ g on day-16 (Fig. 4). The limit for the total viable count which separates the good quality products from bad quality products is 5×10^5 to 10^7

CFU/g (ICMSF 1986). The MRSA counts and TPC showed an inverse relationship. Actively growing bacterial communities in fresh seafood may outcompete MRSA in gaining access to nutrients and these bacteria may also be producing some inhibitory substances which again may inhibit the growth of *S. aureus*.

5.2 Survivability of MRSA in dry Bombay duck stored at room temperature

Dried fish products are at risk from *S. aureus* contamination due to extensive handling involved during their preparation and drying (Moon *et al.*, 2006). The moisture content of dry Bombay duck used in this study was 14.06 %. In dry fish sample spiked with 7 log CFU, the MRSA counts decreased continuously from an initial 5.42 log CFU/g (0-day) to 2.15 log CFU/g on day-20 (Table 20 and Fig. 5). This corresponded to 3.27 log CFU/g or 60% decline in the MRSA count in 20 days at room temperature. Moon *et al.*, (2004) determined the changes in the population of *S. aureus* during 5-month storage of dry filefish and julienned squid. The study showed that the populations of *S. aureus* exhibited reductions of 1.60 log CFU/g in filefish and 0.98 log CFU/g in julienned squid at 10°C. Further, at 24°C *S. aureus* exhibited reductions of 4.45 log CFU/g in filefish, and 5.43 log CFU/g in julienned squid was *S. aureus* and became undetectable in both samples. In the same matrix at 35°C, 6 log CFU/g reduction was observed after 14 days.

Ingham *et al.*, (2006) studied the survival of *S. aureus* in vacuum packaged ready-to-eat meat (dried meat) products with a_w ranging from 0.68 to 0.82 stored at room temperature (21°C). *S. aureus* counts decreased by 1.0 to 2.6 log CFU after 1 week and by 3.2 to 4.5 log CFU after 4 weeks. Erkmén *et al.*, (2008) performed a study on Sucuk (a kind of dry-fermented Turkish sausage by introducing an *S. aureus* contamination level of 3.74 log CFU/g and analyzed for *S. aureus* and aerobic plate count (APC) during manufacturing and storage. Results showed 1.97 and 3.71 log unit decrease in the counts of *S. aureus*.

Another set of dry Bombay duck sample was spiked with 10^4 CFU. The initial contamination level was about 3.56 log CFU/g, and it showed an increase in

initial total contamination level at 4- days sampling was about 5.04 log CFU/g (Table 21). A significant decrease in the count was shown after 12 days sampling which corresponded to 25% of the initial population of MRSA (Fig. 6).

(Erkmen and Bozoglu, (2016) stated that some Gram-positive non-spore-forming bacteria are less sensitive to reduced a_w . Microorganisms present before drying and contaminating during processing can survive for extended periods. If *S.aureus* present before drying, therefore, time and temperature allow them to grow before consumption.

The total plate count in dry Bombay duck showed variations during the whole storage period (Table 23). The initial count was 4.11 log CFU/g (0th day) which increased during the initial storage period increasing to 5.3 log CFU on day-4. The number gradually decreased from day 8 onwards and reached 3.19 on day-20 with a corresponding significant decrease (Fig. 7). The decline was relatively slower compared to MRSA in the same fish.

5.3 Survival rate of MRSA in peeled shrimp and cooked shrimp stored at -20°C

Freezing is widely used to ensure the safety and quality of food. However, previous research has shown that low temperatures are sublethal to *S. aureus*. Studies have shown that at low temperatures, *S. aureus* cells may get damaged, but the bacterium is not killed (Suo *et al*, 2014). Moreover, when the contaminated frozen food is placed at suitable temperatures, the sublethally injured cells are resuscitated and proliferate again. This ability of *S. aureus* makes it a bacterium of concern in low temperature-preserved foods (Wesche *et al*, 2009).

In this study, peeled shrimp spiked with MRSA were stored at -20°C. MRSA survival rate was determined by fortnightly sampling and enumeration on BPA. When inoculated at 7 log CFU level, the counts in peeled shrimp (*Parapenaeopsis stylifera*) decreased from an initial count of 5.44 log CFU/g (day-0) to 3.919 log CFU (day-60) (Table 23 and Fig. 8). The population of MRSA in 60 days declined by 28%.

Statistically significant decline could be noticed after 45 days when the counts decreased to 4.299 from an initial count of 5.44 log CFU (0-day) (Table 23). The total plate counts of MRSA spiked peeled shrimp and stored at -20°C are shown in the (table 25 .), and the changes in the total bacterial populations are shown in the (Fig.10) The TPC decreased from an initial count of 5.11 log CFU/g (day-0) to 3.26 log CFU/g on day-60, which corresponded to 36% decrease in the counts. Unlike in ice-stored fish, in which the total bacterial count increased during storage period, and MRSA counts declined, the trend in frozen stored shrimp was reverse. It may be speculated that the declining total bacterial populations provided better opportunities for MRSA to survive frozen shrimp.

Iyer and Shrivastava (1988) observed 40% and 50% reductions in coagulase-positive *S. aureus* populations in raw shrimps during frozen storage. These investigators observed that the destruction of *S. aureus* in frozen-stored raw shrimps was rapid compared to *S. aureus* in cooked frozen shrimp. In 5 months, the organism became undetectable in raw frozen shrimp compared to 6 months in cooked frozen shrimp (Iyer and Shrivastava 1988). These observations suggest that the survivability of *S. aureus* may depend on the food matrix as well as the test strain itself. Nevertheless, *S. aureus* exhibit significant tolerance to low-temperature shock and freezing cannot alleviate the effects of bad handling of fish and fishery products that introduce *S. aureus*.

Small decrements in *S. aureus* populations (<0.7 log CFU/g) in precooked tuna samples after four weeks of storage at -20 ± 2 °C have been reported (Xulei Wu and Yi-Cheng Su 2014). These investigators further show that *S. aureus* populations increased rapidly once the samples were thawed and held at 35 to 37 °C (Xulei Wu and Yi-Cheng Su 2014). Archer *et al*, (2004) stated that in contrast to the 4°C condition, freezing causes more damage to the cell membrane due to the formation of ice crystals. Damage to the cell membrane leads to the leakage of cellular content, the disruption of membrane permeability and the weakening of the osmoregulation ability of the cell (Wesche *et al*, 2009; Suo *et al*, 2018). This damage also reduces the fluidity of the cell membrane and reduces the enzyme activity, as

shown in a study involving *Listeria monocytogenes* (Schmid *et al*, 2009). Therefore, repair of the cell membrane must occur relatively rapidly upon alleviation of adverse conditions to allow the cells to recover from stress-induced lesions. Suo *et al*, (2014) found that *S. aureus* could survive under freezing (-20°C) condition for longer than 90 days without any further decrease in the viable cell number. All these observations together with the results of this study suggest that the *S. aureus* (MRSA in this study) are generally tolerant to freezing temperatures and their populations are stable after an initial decline immediately after exposure to low temperature.

In peeled shrimp inoculated with 4 log CFU of MRSA, the counts increased from an initial 3.50 log CFU/g (0-day) to 4.35 log CFU/g towards day-15 (24% increase) (Table 23). During subsequent storage, the counts declined and reached 3.73 on day-60 (Fig. 9). These results suggest that MRSA at lower population densities adapts quickly to the freezing environment and its population remains stable. This observation is important because peeled shrimps are subjected to extensive handling leading to contamination by *S. aureus*. However, it could not be determined why the MRSA counts increased during first two weeks of storage. Studies with other food types such as the beef have shown that that population of *S. aureus* decrease slightly by 0.2, 1.1, and 2.0 log CFU/g respectively, after 1, 4, and 7 weeks of storage at -27°C (Erkmen *et al*, 1997).

Bacteria subjected to low temperatures undergo several physiological and structural changes which help them to survive at these temperatures (Beales 2004). However, these abilities are species-specific and it appears that MRSA has better abilities to survive at freezing temperatures in a shrimp environment. Suo *et al*. (2014) reported that freezing temperatures might harm gene transcription and translation leading to the inhibition of cell replication. After the stress of freezing temperature has been alleviated, appropriate signal transduction pathways in the surviving cells must be activated immediately to repair the damage.

In cooked shrimp (*Litopenaeus vannamei*) spiked with 7 log CFU MRSA and stored at -20°C, the survival behaviour of *S. aureus* was found to be different

(Table 26). The count on day-0 was 5.31 log CFU/g which gradually decreased to 3.88 on day-35 (Fig 11). This corresponded to 26% decline from initial population.

A few studies on the behaviour of *S. aureus* in seafood have reported remarkable survivability of this bacterium in fishery products. A seven-fold reduction in the population of *S. aureus* during 130 days of storage at -17.8°C has been reported by Raj Liston (1961). Following this reduction in counts, the population of *S. aureus* remained stable till the end of 393 days of storage (Raj Liston 1961). Iyer and Shrivastava (1988) showed 8 and 15 % destruction of *S. aureus* population in cooked shrimps during freezing at -40°C. These authors also found that there was a gradual decrease in the counts which became undetectable when the products were subsequently stored at -20°C.

Total plate count (TPC) in cooked shrimp on 0- day was 3.63 log CFU/g. The count shows a significant difference after 7- day storage (table 27). The final count decreased about 0.37 log CFU/g and reached about 3.26 log CFU/g (Fig.12). The general effect of cold and freezing on micro-organisms is to diminish the rate of growth, and survival of bacteria in food matrix (Mattick 1951).

In conclusion, this study shows that MRSA survivability in seafood depends on the nature of seafood, temperature of storage as well as the background bacterial flora. *S. aureus* is relatively stable at low temperatures of -20°C and can survive in considerable number even after 60 days. At temperatures which allow rapid multiplication Gram-negative and other Gram-positive bacteria, *S. aureus* growth may be inhibited affecting its long term survivability. Studies are necessary to understand the physiology and *S. aureus* that allows it to survive better at low temperatures and the genetic traits underlying this ability.

The results of real-time quantitative PCR shown in the Table 30 suggested that there was no change in the expression of *lmrS* gene when compared with the expression of 16SrRNA housekeeping gene. The expression was found to be rather low and could not related to the storage temperature or the salt concentrations.

5. DISCUSSION

S. aureus is a versatile pathogen which can grow in a wide range of salt, temperature, water activity and pH (Baeza 2007). Foods that are extensively handled during preparation and kept without refrigeration are usually associated with *Staphylococcal* food poisoning (Kereluk 1961). Humans and animals are natural carriers of *S. aureus* and the contamination of foods with *S. aureus* occurs due to unhygienic handling by food handlers. *S. aureus* does not compete well with other bacteria in foods and hence does not succeed in multiplying in foods when the background bacterial flora is high. Therefore, the presence of *S. aureus* and the consequent food-borne infections by this pathogen is associated with the foods that inhibit the growth of competing microorganisms such as frozen, canned salted, smoked fishery products, boiled fish paste and fish sausages (Bryan, 1980; Sanjeev *et al*, 1986; Nakano *et al*, 2004; Simon and Sanjeev, 2007).

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5.1 Survivability of MRSA in fresh Bombay duck stored in ice

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reached 5.32 log CFU/g on day-14 and again started declining. However, PCR analysis and microscopy revealed that the increase in the counts was due to the growth of *Bacillus paralicheniformis* and *Macrococcus caseolyticus*. The colonies of *M. caseolyticus* were dark black on BPA, but were larger than *S. aureus* and also lacked the halo around the colonies which characteristic of *S. aureus* colonies due to the lecithinase activity (Fig.13). The decrease in MRSA count might be due to the decrease in pH as a result of spoilage of fish and increase in the total bacterial number that *S. aureus* may not be able to compete with (Farrell and Upton 1978).

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In fresh Bombay duck spiked with 4 log CFU of MRSA, the initial count was 3.29 log CFU/g on day-0. Interestingly, the total MRSA count increased by 2.07 log CFU/g and reached 5.36 log CFU/g on day-6 (Table 17). From day-8, the MRSA counts showed decreasing trend and reached 3.70 log CFU at the end of the storage period (day-18) (Fig.3). The results suggest that the behaviour of MRSA in ice-stored fish is different when present in different population densities. When MRSA population is in the range of 3 or 4 log CFU, the bacterium is able to adapt well, obtain nutrients and survive in the chilled condition in Bombay duck. Apart from the observed population dynamics of MRSA, complex interactions among *S. aureus* and between *S. aureus* and other bacteria in fish may exist.

The initial total plate count (TPC) of freshly Bombay duck was about 4.0 log CFU/g (Table 18) and during storage in ice, the counts continuously increased and reached 7.32 log CFU/ g on day-16 (Fig. 4). The limit for the total viable count which separates the good quality products from bad quality products is 5×10^5 to 10^7

CFU/g (ICMSF 1986). The MRSA counts and TPC showed an inverse relationship. Actively growing bacterial communities in fresh seafood may outcompete MRSA in gaining access to nutrients and these bacteria may also be producing some inhibitory substances which again may inhibit the growth of *S. aureus*.

5.2 Survivability of MRSA in dry Bombay duck stored at room temperature

Dried fish products are at risk from *S. aureus* contamination due to extensive handling involved during their preparation and drying (Moon *et al.*, 2006). The moisture content of dry Bombay duck used in this study was 14.06 %. In dry fish sample spiked with 7 log CFU, the MRSA counts decreased continuously from an initial 5.42 log CFU/g (0-day) to 2.15 log CFU/g on day-20 (Table 20 and Fig. 5). This corresponded to 3.27 log CFU/g or 60% decline in the MRSA count in 20 days at room temperature. Moon *et al.*, (2004) determined the changes in the population of *S. aureus* during 5-month storage of dry filefish and julienned squid. The study showed that the populations of *S. aureus* exhibited reductions of 1.60 log CFU/g in filefish and 0.98 log CFU/g in julienned squid at 10°C. Further, at 24°C *S. aureus* exhibited reductions of 4.45 log CFU/g in filefish, and 5.43 log CFU/g in julienned squid was *S. aureus* and became undetectable in both samples. In the same matrix at 35°C, 6 log CFU/g reduction was observed after 14 days.

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Another set of dry Bombay duck sample was spiked with 10^4 CFU. The initial contamination level was about 3.56 log CFU/g, and it showed an increase in

initial total contamination level at 4- days sampling was about 5.04 log CFU/g (Table 21). A significant decrease in the count was shown after 12 days sampling which corresponded to 25% of the initial population of MRSA (Fig. 6).

(Erkmen and Bozoglu, (2016) stated that some Gram-positive non-spore-forming bacteria are less sensitive to reduced a_w . Microorganisms present before drying and contaminating during processing can survive for extended periods. If *S.aureus* present before drying, therefore, time and temperature allow them to grow before consumption.

The total plate count in dry Bombay duck showed variations during the whole storage period (Table 23). The initial count was 4.11 log CFU/g (0th day) which increased during the initial storage period increasing to 5.3 log CFU on day-4. The number gradually decreased from day 8 onwards and reached 3.19 on day-20 with a corresponding significant decrease (Fig. 7). The decline was relatively slower compared to MRSA in the same fish.

5.3 Survival rate of MRSA in peeled shrimp and cooked shrimp stored at -20°C

Freezing is widely used to ensure the safety and quality of food. However, previous research has shown that low temperatures are sublethal to *S. aureus*. Studies have shown that at low temperatures, *S. aureus* cells may get damaged, but the bacterium is not killed (Suo *et al*, 2014). Moreover, when the contaminated frozen food is placed at suitable temperatures, the sublethally injured cells are resuscitated and proliferate again. This ability of *S. aureus* makes it a bacterium of concern in low temperature-preserved foods (Wesche *et al*, 2009).

In this study, peeled shrimp spiked with MRSA were stored at -20°C. MRSA survival rate was determined by fortnightly sampling and enumeration on BPA. When inoculated at 7 log CFU level, the counts in peeled shrimp (*Parapenaeopsis stylifera*) decreased from an initial count of 5.44 log CFU/g (day-0) to 3.919 log CFU (day-60) (Table 23 and Fig. 8). The population of MRSA in 60 days declined by 28%.

Statistically significant decline could be noticed after 45 days when the counts decreased to 4.299 from an initial count of 5.44 log CFU (0-day) (Table 23). The total plate counts of MRSA spiked peeled shrimp and stored at -20°C are shown in the (table 25 .), and the changes in the total bacterial populations are shown in the (Fig.10) The TPC decreased from an initial count of 5.11 log CFU/g (day-0) to 3.26 log CFU/g on day-60, which corresponded to 36% decrease in the counts. Unlike in ice-stored fish, in which the total bacterial count increased during storage period, and MRSA counts declined, the trend in frozen stored shrimp was reverse. It may be speculated that the declining total bacterial populations provided better opportunities for MRSA to survive frozen shrimp.

Iyer and Shrivastava (1988) observed 40% and 50% reductions in coagulase-positive *S. aureus* populations in raw shrimps during frozen storage. These investigators observed that the destruction of *S. aureus* in frozen-stored raw shrimps was rapid compared to *S. aureus* in cooked frozen shrimp. In 5 months, the organism became undetectable in raw frozen shrimp compared to 6 months in cooked frozen shrimp (Iyer and Shrivastava 1988). These observations suggest that the survivability of *S. aureus* may depend on the food matrix as well as the test strain itself. Nevertheless, *S. aureus* exhibit significant tolerance to low-temperature shock and freezing cannot alleviate the effects of bad handling of fish and fishery products that introduce *S. aureus*.

Small decrements in *S. aureus* populations (<0.7 log CFU/g) in precooked tuna samples after four weeks of storage at -20 ± 2 °C have been reported (Xulei Wu and Yi-Cheng Su 2014). These investigators further show that *S. aureus* populations increased rapidly once the samples were thawed and held at 35 to 37 °C (Xulei Wu and Yi-Cheng Su 2014). Archer *et al*, (2004) stated that in contrast to the 4°C condition, freezing causes more damage to the cell membrane due to the formation of ice crystals. Damage to the cell membrane leads to the leakage of cellular content, the disruption of membrane permeability and the weakening of the osmoregulation ability of the cell (Wesche *et al*, 2009; Suo *et al*, 2018). This damage also reduces the fluidity of the cell membrane and reduces the enzyme activity, as

shown in a study involving *Listeria monocytogenes* (Schmid *et al*, 2009). Therefore, repair of the cell membrane must occur relatively rapidly upon alleviation of adverse conditions to allow the cells to recover from stress-induced lesions. Suo *et al*, (2014) found that *S. aureus* could survive under freezing (-20°C) condition for longer than 90 days without any further decrease in the viable cell number. All these observations together with the results of this study suggest that the *S. aureus* (MRSA in this study) are generally tolerant to freezing temperatures and their populations are stable after an initial decline immediately after exposure to low temperature.

In peeled shrimp inoculated with 4 log CFU of MRSA, the counts increased from an initial 3.50 log CFU/g (0-day) to 4.35 log CFU/g towards day-15 (24% increase) (Table 23). During subsequent storage, the counts declined and reached 3.73 on day-60 (Fig. 9). These results suggest that MRSA at lower population densities adapts quickly to the freezing environment and its population remains stable. This observation is important because peeled shrimps are subjected to extensive handling leading to contamination by *S. aureus*. However, it could not be determined why the MRSA counts increased during first two weeks of storage. Studies with other food types such as the beef have shown that that population of *S. aureus* decrease slightly by 0.2, 1.1, and 2.0 log CFU/g respectively, after 1, 4, and 7 weeks of storage at -27°C (Erkmen *et al*, 1997).

Bacteria subjected to low temperatures undergo several physiological and structural changes which help them to survive at these temperatures (Beales 2004). However, these abilities are species-specific and it appears that MRSA has better abilities to survive at freezing temperatures in a shrimp environment. Suo *et al*. (2014) reported that freezing temperatures might harm gene transcription and translation leading to the inhibition of cell replication. After the stress of freezing temperature has been alleviated, appropriate signal transduction pathways in the surviving cells must be activated immediately to repair the damage.

In cooked shrimp (*Litopenaeus vannamei*) spiked with 7 log CFU MRSA and stored at -20°C, the survival behaviour of *S. aureus* was found to be different

(Table 26). The count on day-0 was 5.31 log CFU/g which gradually decreased to 3.88 on day-35 (Fig 11). This corresponded to 26% decline from initial population.

A few studies on the behaviour of *S. aureus* in seafood have reported remarkable survivability of this bacterium in fishery products. A seven-fold reduction in the population of *S. aureus* during 130 days of storage at -17.8°C has been reported by Raj Liston (1961). Following this reduction in counts, the population of *S. aureus* remained stable till the end of 393 days of storage (Raj Liston 1961). Iyer and Shrivastava (1988) showed 8 and 15 % destruction of *S. aureus* population in cooked shrimps during freezing at -40°C. These authors also found that there was a gradual decrease in the counts which became undetectable when the products were subsequently stored at -20°C.

Total plate count (TPC) in cooked shrimp on 0- day was 3.63 log CFU/g. The count shows a significant difference after 7- day storage (table 27). The final count decreased about 0.37 log CFU/g and reached about 3.26 log CFU/g (Fig.12). The general effect of cold and freezing on micro-organisms is to diminish the rate of growth, and survival of bacteria in food matrix (Mattick 1951).

In conclusion, this study shows that MRSA survivability in seafood depends on the nature of seafood, temperature of storage as well as the background bacterial flora. *S. aureus* is relatively stable at low temperatures of -20°C and can survive in considerable number even after 60 days. At temperatures which allow rapid multiplication Gram-negative and other Gram-positive bacteria, *S. aureus* growth may be inhibited affecting its long term survivability. Studies are necessary to understand the physiology and *S. aureus* that allows it to survive better at low temperatures and the genetic traits underlying this ability.

The results of real-time quantitative PCR shown in the Table 30 suggested that there was no change in the expression of *lmrS* gene when compared with the expression of 16SrRNA housekeeping gene. The expression was found to be rather low and could not related to the storage temperature or the salt concentrations.

6. SUMMARY

Staphylococcus aureus is an important bacterial agent of food poisoning. The emergence of methicillin-resistant *S. aureus* (MRSA) has raised concerns about the contamination of foods with this bacterium. The physiology of bacterial survival and persistence in foods is of considerable research interest since this information can be used to control or eliminate pathogenic and food poisoning bacteria from the food. In this respect, the present study was conducted to determine the survivability of methicillin-resistant *S. aureus* in different fish and fishery products, and to understand the role of the *ImrS* gene in the survivability of MRSA in fish. Fresh Bombay duck (*Harpodon nehereus*), dried Bombay duck, fresh shrimp (*Parapenaeopsis styliifera*) and cooked shrimp (*Litopenaeus vannamei*) were used for spiking with *S. aureus*.

The experiments with *S. aureus* were done at two different inoculation levels, representing low populations of *S. aureus* (10^4 CFU) and high populations of *S. aureus* (10^7 CFU) in seafood. *S. aureus* counts were determined on Baird Parker Agar (BPA), while the total bacterial count was determined on Plate Count Agar (PCA). Fresh Bombay duck was stored in ice for 16 to 18 days, and the bacterial enumeration was done on alternate days. Dry Bombay duck was stored at room temperature ($30^\circ\text{C}\pm 3^\circ$) for 20 days, and sampling was done at 4- days interval. Cooked and peeled shrimp were stored frozen at -20°C and were sampled for bacterial enumeration at weekly and fortnightly intervals, respectively.

In fresh Bombay duck stored in ice, the total plate count (TPC) reached 10^7 log CFU/gm at the end of the storage study (day-16). The survival behaviours of MRSA was found to be different in fresh fish at different population densities. In fish inoculated with 4 log CFU, MRSA counts increased initially till day-6 and gradually declined thereafter and remained stable at 3 log CFU till the end of storage period. On the contrary, MRSA inoculated at 7 log CFU declined by 48% of the initial population and reached 3.51 log CFU at the end of storage period. In dry Bombay duck inoculated with 7 log CFU, the MRSA counts decreased continuously from an initial 5.42 log CFU/g (0-day) to 2.15 log CFU/g on day-20 which corresponded to 60% decline in the

MRSA count in 20 days at room temperature. Unlike in iced fish, the total bacterial count of dry Bombay duck increased initially from 4.11 log CFU/g to 5.3 log CFU/g and subsequently decreased to 3.19 log CFU on day-20.

In peeled shrimp stored at -20°C and inoculated with 7 log CFU, the counts of MRSA declined by 28% in 60 days, from an initial count of 5.44 log CFU/g to 3.919 log CFU/g. However, at lower population densities of 10⁴ CFU, the counts of MRSA remained stable at 3.73 log CFU after 60 days. The TPC decreased from an initial count of 5.11 log CFU/g (day-0) to 3.26 log CFU/g on day-60, which corresponded to 36% decrease in the counts. In cooked shrimp (*Litopenaeus vannamei*) spiked with 7 log CFU MRSA and stored at -20°C, the count declined from 5.31 log CFU/g to 3.81 by the end of 35 day storage period, which corresponded to 28% decline in MRSA population.

The expression of *lmrS* efflux pump was studied using real-time quantitative (RT) PCR to determine if this pump has any role in the survivability of MRSA at different storage temperatures (chilled and -20°C) and salt concentrations (5%, 10%, 15% NaCl). RT-PCR results did not reveal any significant differences in the expression of a *lmrS* gene at different temperature and salt conditions.

In conclusion, the survivability of MRSA in seafood differs depending on the inoculation matrix, storage temperature, changes in the populations of background bacteria in fish and the population density of MRSA itself. Overall, MRSA displayed better survivability in shrimp stored at -20°C, especially at lower population densities of less than 4 log CFU. These results suggest that -20°C or similar freezing temperatures are not lethal to MRSA and its number may increase subsequently during thawing and food preparation. Future studies should investigate the effect of long-term low-temperature storage on the survivability of MRSA in comparison with methicillin-sensitive *S. aureus* and the genetic features responsible for the low-temperature survivability of MRSA in low temperature preserved fish and fishery products.

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