Research Article

Antibiotic Resistance and Virulence Gene Characteristics of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolated from Healthy Edible Marine Fish

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Abstract

Thirty-three (33) isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) from healthy edible marine fish harvested from two aquaculture settings and the Kariega estuary, South Africa, were characterised in this study. The phenotypic antimicrobial susceptibility profiles to 13 antibiotics were determined, and their antibiotic resistance determinants were assessed. A multiplex PCR was used to determine the epidemiological groups based on the type of SCCmec carriage followed by the detection of staphylococcal enterotoxin-encoding genes sea-sea and the Panton Valentine leucocidin gene (pvl). A high antibiotic resistance percentage (67–81%) was observed for Erythromycin, Ampicillin, Rifampicin, and Clindamycin, while maximum susceptibility to Chloramphenicol (100%), Imipenem (100%), and Ciprofloxacin (94%) was recorded. Nineteen (58%) of the MRSA strains had Vancomycin MICs of ≤2 μg/mL, 4 (12%) with MICs ranging from 4–8 μg/mL, and 10 (30%) with values ≥16 μg/mL. Overall, 27 (82%) isolates were multidrug-resistant (MDR) with Erythromycin-Ampicillin-Rifampicin-Clindamycin (E-AMP-RIP-CD) found to be the dominant antibiotic-resistance phenotype observed in 4 isolates. Resistance genes such as tetM, tetA, ermB, blaZ, and femA were detected in two or more resistant strains. A total of 19 (58%) MRSA strains possessed SCCmec types I, II, or III elements, characteristic of healthcare-associated MRSA (HA-MRSA), while 10 (30%) isolates displayed SCCmec type IVc, characteristic of community-associated MRSA (CA-MRSA). Six (18%) of the multidrug-resistant strains of MRSA were enterotoxigenic, harbouring the see, sea, or sec genes. A prevalence of 18% (6/33) was also recorded for the luk-PVL gene. The findings of this study showed that marine fish contained MDR-MRSA strains that harbour SCCmec types characteristic of either HA-MRSA or CA-MRSA, but with a low prevalence of enterotoxin and pvl genes. Thus, there is a need for continuous monitoring and implementation of better control strategies within the food chain to minimise contamination of fish with MDR-MRSA and the ultimate spread of the bug.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major opportunistic pathogen known to cause severe multidrug-resistant infections in animals and humans. It is also a significant foodborne pathogen due to its staphylococcal enterotoxin (SE) producing abilities. These strains result from *S. aureus* that acquire the mecA gene carried on an integrated staphylococcal cassette chromosome mec (SCCmec). The gene encodes a 78-kDa Penicillin-binding protein (PBP2a) with a lower affinity to Methicillin and other beta-lactam antibiotics [1].

Clinicians often face a challenge in treating MRSA infections due to their marked resistance to various classes of antibiotics. The success of this pathogen is related to the remarkable ability of *S. aureus* to quickly adapt and acquire resistance to multiple antibiotics introduced in clinical practice over the years, coupled with its extensive battery of
virulence factors [2]. Infections caused by S. aureus vary in severity from minor skin infections, such as boils, carbuncles, pimpls, abscesses, cellulitis, folliculitis, impetigo, and scalded skin syndrome, to life-threatening conditions, such as bacteraemia, endocarditis, meningitis, toxic shock syndrome, pneumonia, and osteomyelitis. More than 80% of mortality rate was recorded for life-threatening infections caused by the pathogen until the development of Penicillin in the 1940s, which significantly improved therapy [3, 4]. Unfortunately, this medical success was short-lived as nosocomial infections were associated with increased frequency of resistance to Penicillin conferred by the production of a plasmid-located beta-lactamase gene, blaZ. The introduction of the first semisynthetic Penicillin, Methicillin, in 1959, later proved ineffective as there also emerged hospital-associated MRSA (HA-MRSA), most limited to health care settings [4]. MRSA was later disseminated to the community with the first virulent community-associated MRSA (CA-MRSA) reported in the late 1990s, which possessed the virulent Panton Valentine leucocidin (PVL) toxin [5].

Management of MRSA infections includes the use of Vancomycin, one of the essential drugs of choice for invasive infections. Other antibiotics, such as Clindamycin, Trimethoprim-sulamethoxazole and Tetracycline, are also used, with some offered in combination with a beta-lactam such as Amoxicillin or with other antibiotics such as Cefazolin, Linezolid, or Rifampicin [6]. Generally, MRSA strains have been associated with increased incidences of resistance to other antibiotics. This has been attributed not only to the global trend of antibiotic resistance due to over-and indiscriminate use, but also to the unique ability of the pathogen to easily acquire resistance [7–9].

MRSA has been clustered into different epidemiological groups based on the type of SCCmec element carriage and the virulence related to the PVL toxin. These include HA-MRSA, CA-MRSA, and livestock-associated-MRSA (LA-MRSA). While CA-MRSA is associated with smaller SCCmec elements (types IV and V) and often harbours the potent PVL toxin, HA-MRSA is characterised by a larger SCCmec element (I, II, III) and less frequently produces the PVL toxin [10–12]. This toxin can penetrate undamaged skin causing severe infections. It is thought to have evolved from community-associated methicillin susceptible S. aureus strains due to the transmission of the pvl gene on bacteriophages [11]. Although the reservoir of CA-MRSA is rapidly expanding, compared to nosocomial strains, CA-MRSA strains are more susceptible to a variety of antibiotics and resistant to a few classes, frequently, the beta-lactams and macrolides [12–14]. Livestock-associated MRSA (LA-MRSA), on the other hand, is commonly found in animals with differing genetic backgrounds from those of CA-MRSA and HA-MRSA [15].

MRSA is able to produce one or more staphylococcal enterotoxins (SEs), which are part of the main virulence factors of the pathogen. These toxins possess potent superantigenic activity and are composed of the classical (SEA-SEE) and newer (SEG-SEY) types [16]. Members of these SEs play a vital role in outbreaks of food poisoning and other infections that are septic-related [16]. Staphylococcal enterotoxins are heat stable and, therefore, are able to thrive and maintain their activity in food previously contaminated with the pathogen. In their review of MRSA as a foodborne pathogen, Wendlandt et al. [17] conclude that CA-MRSA, LA-MRSA, and HA-MRSA can be present in food and food products meant for human consumption. Although there is no evidence of the direct transmission of MRSA from fish to humans, interaction with the aquatic environment as well as their involvement in handling and consumption of marine food, provides a possibility that MRSA contaminated fish can be a source of foodborne infection to humans. Larsen et al. [18] reported one of the first incidences of food animals as a source of human MRSA infection. The researchers identified a new strain of poultry-associated MRSA in humans in Denmark, without any prior exposure to livestock and, therefore, were most likely acquired by eating or handling contaminated poultry meat. The genetic analysis of the strains linked them to imported poultry strains from other European countries.

There is available data on the occurrence and molecular characteristics of MRSA in humans and other animals. Also, in the last decade, investigation of MRSA in food and food-producing animals has received considerable attention. On the contrary, few studies have focused on MRSA in fish [19–29]. The majority of these studies have focused on the prevalence or incidence of the superbug, with no further characterisation. There is, therefore, an information gap with regard to the characteristics of MRSA from fish. Also, over 99% of previous reports of MRSA from fish have focused on the incidence from wild catch compared to the current study, where over 97% of the isolates were from tank cultured edible marine fish. This study, therefore, focused on antimicrobial resistance, virulence profiles, and possible epidemiological types of MRSA isolated from edible marine fish.

2. Materials and Methods

2.1. Bacterial Isolates. Thirty-three (33) MRSA isolates were characterised in this study. These isolates were recovered from 100 aquaculture and 20 wild marine fish from an earlier study, which represented the first report of the detection of MRSA in marine aquaculture fish in South Africa [29]. Thirty-two (32) isolates were recovered from aquaculture fish while one was isolated from wild catch. Methicillin resistance was previously determined using Cefoxitin and Oxacillin, followed by PCR detection of the meca gene [29]. All strains were maintained in Tryptic Soy Broth (TSB) supplemented with 25% glycerol and stored at −80°C.

2.2. Antibiotic Susceptibility Testing. MRSA isolates were recovered from glycerol stocks by plating on nutrient agar and incubating at 37°C for 24 hours. The isolates were subjected to antibiotic susceptibility testing (12 antibiotics belonging to 9 classes) using the disk diffusion assay on Muller Hinton Agar (MHA) in accordance with the guidelines of the Clinical and Laboratory Standards Institute.
Doxycycline (30 μg), Erythromycin (15 μg), Gentamicin (10 μg), Levofloxacin (5 μg), Clindamycin (2 μg), Imipenem (10 μg), Ciprofloxacin (5 μg), Tetracycline (30 μg), and Trimethoprim-sulfamethoxazole (1.25/23.75 μg) were dispensed on inoculated plates using a Mast discs dispenser (Mast diagnostics, UK). Colonies from an overnight pure culture were transferred to a microtiter plate, and twofold serial dilutions were performed in 25 μL template DNA, 0.5 μL of each oligonucleotide, 12.5 μL of 2X PCR master mix (Bio Labs, New England), and an appropriate volume of nuclelease-free water (Bio Labs, New England). The resulting amplicons were resolved by gel electrophoresis in a 1.5% (w/v) agarose gel run at 100 volts for 40 minutes in a 0.5XTAE buffer. The gels were visualised under a UV trans-illuminator (Alliance 4.7 XD-79, Uvitec, Cambridge, UK).

3. Results

3.1. Antimicrobial Resistance Profile of MRSA. All MRSA isolates were susceptible to Gentamicin, Chloramphenicol, and Imipenem. On the other hand, the highest antibiotic resistance (82%) was to Rifampicin and Clindamycin, followed by Erythromycin and Ampicillin with 67% (22/33) resistance each. Twelve strains (36%) were resistant to Doxycycline, 10 (30%) to Trimethoprim-Sulfamethoxazole, and 9 (27%) to Tetracycline. Only one (3.0%) strain was resistant to Ciprofloxacin. Figure 1 shows the resistance profiles of MRSA isolates assayed using the disk diffusion method.

3.2. Vancomycin MICs. The MIC for Vancomycin with values of ≤2 μg/mL was recorded in 58% (19/33) of the MRSA strains considered as susceptible, 12% (n = 4) with MICs range of 4–8 μg/mL (intermediate), and 30% (n = 10) with values of ≥16 μg/mL (resistant).

3.3. Multiple Antibiotic Resistance (MAR). Most (82%, n = 27) of the MRSA strains were multiple antibiotic-resistant (resistant to three or more antibiotics of at least 2 classes) with MAR index greater than 0.2. A total of 20 MAR
phenotypes were obtained with two strains exhibiting resistance of up to 8 (E-AMP-RIP-DO-CD-VA-TE-SXT) of the 13 antimicrobials tested. The phenotype E-AMP-RIP-CD was dominant (n = 4), followed by E-AMP-RIP-DO-CD-VA-SXT (n = 3). For all isolates that were resistant to Vancomycin (n = 10), at least five other antibiotics were considered ineffective therapies for their in vitro inhibition (Table 3).

3.4. Prevalence of Antibiotic Resistance Genes. The presence of femA genes among MRSA isolates was at a frequency of 85% (28/33), while 59% (13/22) of blaZ-positive were recorded from β-lactam-resistant strains. The ermB gene was present in 7 (32%; 7/22) of Erythromycin-resistant isolates, while tetM and tetA were recorded in 89% (8/9) and 22% (2/9), respectively, for Tetracycline-resistant strains. Antibiotic resistance determinants ermA and ermC were not detected in the study.

3.5. Occurrence of Enterotoxin Genes, SCCmec Types, and pvl. Six (18%) isolates were enterotoxigenic, all of which were MDR strains: enterotoxin e was detected in 4 strains (12%), followed by sea (3%) and sec (3.0%) detected in one strain each. A multiplex PCR for SCCmec typing revealed a total of 19 (58%) MRSA strains that possessed SCCmec types I (n = 1), II (n = 1) or III (n = 17) characteristic of HA-MRSA while 10 (30%) carried the SCCmec type IVc element

| Gene target | Oligonucleotide sequences (5′-3′) | Product size (bp) | Annealing temperature (°C) | References |
|-------------|----------------------------------|-------------------|---------------------------|------------|
| ermA        | F: TATCTATCGTTGAGAAAGGGATT R: TATCTATCGTTGAGAAAGGGATT | 139               | 47.6                      |            |
| ermB        | F: CTATCTGATTGTGAGAAAGGGATT R: CTATCTGATTGTGAGAAAGGGATT | 142               | 47                        | [34]       |
| ermC        | F: CTATCTGATTGTGAGAAAGGGATT R: CTATCTGATTGTGAGAAAGGGATT | 190               | 47                        | [34]       |
| blaZ        | F: CTATCTGATTGTGAGAAAGGGATT R: CTATCTGATTGTGAGAAAGGGATT | 173               | 49                        |            |
| tetA        | F: GCTACATCCGTTGCTGGTCCTTC R: ATAGATCGCCGTTGAGAAGGG | 209               | 50                        | [35]       |
| tetM        | F: AGTGGAGCGATTACAGAA R: CAT ATG TCC TGG C GTG TGC TA | 158               | 45                        | [36]       |
| femA        | F: AAAAAGCATAAAACAGG G R: GATAAAAGAAGAAACAGGAGAAGG | 132               | 45.5                      | [37]       |

Table 2: Oligonucleotide sequences used for the detection of staphylococcal enterotoxins, pvl, and SCCmec types.

| Gene target/specificity | Sequence (5′-3′) | Product size (bp) | Reference |
|-------------------------|------------------|-------------------|-----------|
| Universal fw            | F: TATCTATCGTTGAGAAAGGGATT | —                | [38]      |
| Sea                     | R: ATTACCAAGGGGTATCGT         | 270               |           |
| Seb                     | R: ATAGATCGCCGATTAGAAGGGATT  | 165               |           |
| Sec                     | R: AATTACACATTTGTGAGAAGGGATT | 69                | [38]      |
| Sed                     | R: ATGATCGCCGATTAGAAGGGATT  | 306               |           |
| Sec                     | R: GCTACATCCGTTGCTGGCTCAGG   | 213               |           |
| Luk-PVL                 | F: ATCATTAGGTAATAGTCTGAGAAGGGAGG | 433              | [39]      |
| SCCmec I                | F: GCTATTGAGAAGGTGTTAGGACGG  | 613               |           |
| SCCmec II               | R: GTTCCTCTCAGATGACAGGTGTC   | 398               |           |
| SCCmec III              | R: CGGTTAACATCAGAAGGGAGGG    | 280               |           |
| SCCmec Iva              | R: ATGATCGCCGATTAGAAGGGATT  | 776               |           |
| SCCmec IVb              | R: AATGATCGCCGATTAGAAGGGATT | 493               | [40]      |
| SCCmec IVc              | F: ACAATATTTTGTATTACTGAGGAGGC | 200               |           |
| SCCmec IVd              | R: TTGATTGAGTATTACTGAGGAGGC  | 881               |           |
| SCCmec V                | R: GCTACATCCGTTGCTGGCTCAGG   | 325               |           |

Table 1: Primer sequences used for the detection of antibiotic resistance genes.
characteristic of CA-MRSA. SCCmec type V was not detected and four strains could not be typed. Six (18%) of the 33 MRSA isolates were *pvl* positive distributed as follows: one SCCmec type I strain; two SCCmec type III strain; and three SCCmec type IVc strain.

### 4. Discussion

Antibiotic susceptibility testing revealed Gentamicin, Imipenem, Chloramphenicol, and Ciprofloxacin as the most effective antimicrobials for MRSA, while high resistance to Rifampicin, Clindamycin, Ampicillin, and Erythromycin were observed. Over 80% of the samples were recorded as MDR strains. The high individual resistance to certain antimicrobials and the prevalence of MDR strains are not surprising as multidrug resistance is becoming a typical feature of MRSA, coupled with global antimicrobial resistance challenge [9, 41, 42]. The results obtained in this study are comparable with high levels of up to 100% MDR-MRSA recorded from pigs and retail food in China [43] as well as from poultry and farm workers in South Africa [44]. However, a 37.2% (29/78) prevalence of multidrug-resistant MRSA from retail meat in the United States is in contrast to our findings [45]. High individual resistances of MRSA to

![Figure 1: Antimicrobial-resistant patterns of 33 MRSA isolates to 12 antibiotics tested using the disk diffusion assay.](chart)

**Table 3: Phenotypes of MDR-MRSA isolates.**

| No. of antibiotics resistant | Resistant phenotype                      | Frequency of occurrence |
|-----------------------------|------------------------------------------|-------------------------|
| 3                           | AMP-RIP-CD                               | 1                       |
|                             | E-RIP-CD                                 | 1                       |
|                             | E-DO-TE                                  | 1                       |
|                             | E-RIP-DO                                 | 1                       |
|                             | DO-CD-TE                                 | 1                       |
|                             | DO-CD-TE-SXT                             | 1                       |
|                             | E-AMP-RIP-CD                             | 1                       |
|                             | AMP-RIP-DO-CD                            | 1                       |
| 4                           | AMP-RIP-CD-TE                            | 1                       |
|                             | E-AMP-RIP-CD-CD-TE                       | 1                       |
|                             | E-AMP-RIP-CD-SXT                         | 1                       |
|                             | E-AMP-RIP-CD-VA-TE                       | 1                       |
| 5                           | E-AMP-RIP-CD-VA-TE-VA-TE                 | 1                       |
| 6                           | E-AMP-RIP-DO-CD-TE                       | 1                       |
|                             | E-AMP-RIP-DO-CD-VA-SXT                   | 1                       |
| 7                           | E-AMP-RIP-DO-CD-VA-SXT                   | 1                       |
|                             | E-AMP-RIP-DO-CD-VA-VA-TE-SXT             | 1                       |
| 8                           | E-AMP-RIP-DO-CD-VA-TE-VA-TE-SXT          | 1                       |

C: Chloramphenicol; E: Erythromycin; AMP: Ampicillin; RIP: Rifampicin; DO: Doxycycline; GN: Gentamicin; LEV: Levofloxacin; CD: Clindamycin; IPM: Imipenem; CIP: Ciprofloxacin; TE: Tetracycline, and SXT: Trimethoprim-Sulfamethoxazole.
Rifampicin (82%), Clindamycin (82%), Erythromycin (67%), and Ampicillin 67% are similar to those recorded by Sato et al. [46], although the percent Tetracycline resistance in our study was much lower (27%) than previously reported (100%) from pigs in Japan [46]. The resistance of isolates to Vancomycin was 30%. This is quite disturbing as Vancomycin represents the standard therapy for invasive MRSA infections in humans [47, 48]. Vancomycin-resistant MRSA has also been detected in camel meat, whereby all isolates detected as Vancomycin-resistant S. aureus were MRSA strains [49]. Other studies have revealed the emergence of Vancomycin-resistant MRSA strains in other parts of the world, since its first detection in 1996 in a hospital case in Japan, and in the US in 2002 [47, 50]. The increased frequency of resistance of MRSA to this antibiotic suggests the need for combination therapy or adoption of alternatives or inclusion of newer regimens for the treatment of invasive MRSA infections.

Antibiotic resistance determinants were prevalent in MRSA isolates and included the following: femA, blaZ, tetA, tetM, and ermB. The chromosomally located femA gene has been shown to encode proteins that significantly affect the level of Methicillin resistance in S. aureus thus, probably the reason for its high prevalence in the MRSA strains in this study. PCR detection of this gene, in addition to mecA detection, has been proposed as a reliable indicator of MRSA that easily differentiates it from mecA-positive coagulase-negative staphylococci [51]. Resistance genes, such as tetA, tetM, and ermB reported from our isolates, have also been reported in MRSA isolated from poultry and farm workers in South Africa [44] while tetM and ermB were reported in clinical isolates [52]. More antibiotic-resistant bacterial strains are continually reported due to increased use of the same or similar antibiotics in aquaculture, animal husbandry, and in the management of human diseases. Also, large-scale settings of aquaculture have led to increased antibiotic resistance in bacteria that are potentially pathogenic to both fish and humans [53].

One of the most common seafood-borne intoxications is due to preformed SEs in food, thus resulting in outbreaks of foodborne diseases around the world. Of these, staphylococcal enterotoxin A, followed by D, is the most frequently associated with these outbreaks, although the other classical type toxins, together with SEH, have also been reported [54]. These toxins are also involved in toxic shock syndrome and other staphylococcal infections. In the present study, a total of 6 MRSA strains were recorded as positive for genes encoding the classical type SEs, with see detected in four of the strains. These results are contrary to other studies with higher frequencies of enterotoxigenic MRSA [55–57]. However, this is the first report that focused on the detection of toxin genes in MRSA strains isolated from marine fish in South Africa. The most frequently detected gene was see, which is in line with the study by Arfatahery et al. [57]. Growth of Staphylococcus sp. and production of enterotoxin in food have been reported to be aided by poor personal hygiene, inadequate refrigeration, delays in processing, and postprocess contamination [54]. The low levels of these enterotoxins in the present study, if not intrinsic, could be due to minimal delay time between sample collection and analysis. Just a few handlers were in contact with the fish samples. However, continuous food monitoring is still required to minimise contamination with enterotoxigenic MRSA that can occur between fish harvesting, throughout processing to the final product.

The types of SCCmec detected were I, II, III, and IV although I and II were detected at a relatively lower frequency to types III (n = 17) and IV (n = 10). This is contrary to the findings where the bulk of SCCmec from MRSA isolates detected from processed food in Pakistan were type IV followed by types II and III [58]. Type IV MRSA strains have also been isolated from retail meat and humans in Georgia [59]. Studies have shown that SCCmec types I–III are mostly associated with HA-MRSA, while types IV and V are linked to CA-MRSA [10–12, 60, 61]. The results, therefore, are an indication that over 57% of strains in the current study were likely to be HA-MRSA while 30% were CA-MRSA. This is an indication that fish is a potential reservoir for HA- and CA-MRSA to humans. Four strains could not be typed and were considered to belong to other SCCmec types/subtypes not included in the screen.

The PVL toxin has gained considerable attention as one of the major virulence factors present in CA-MRSA strains [12, 13, 41, 62]. Consequently, it has been used as an epidemiological tool to determine the nature of MRSA, whether it is CA-MRSA or HA-MRSA. A low frequency of pvl (18.2%; 6/33) was recorded in this study, although higher than studies which revealed the absence of the gene in isolates from retail raw fish and raw meat samples [24, 56]. This gene is mostly associated with CA-MRSA strain, which is reflected in the study as 16% (3/19) of HA-MRSA possessed the pvl gene compared to 30% (3/10) of CA-MRSA strains. Sivaraman et al. [63] also found the pvl gene in approximately 16% of CA-MRSA from seafood in India, although lower than the percentage recorded in this study.

5. Conclusions
This study revealed a wide range of multiple antibiotic-resistance profiles of MRSA isolated from marine fish. Although the prevalence of toxin genes was low, food poisoning from infection with such strains cannot be ruled out. Thus, there is a need for continuous and better control of sources of food contamination and the spread of antimicrobial-resistant bacteria since the pathogenic potential of these strains cannot be ignored.

Data Availability
The data used to support the findings of this study are included in the article.

Conflicts of Interest
The authors declare no conflicts of interest.

Authors’ Contributions
Justine Fri conceptualised the study, performed the experiments, analysed and interpreted the data, and wrote the
manuscript. Roland Ndpi Ndpi conceptualised and supervised the experiments. Henry Akum Njom and Collins Njie Ateba proofread, reviewed, and edited the manuscript.

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