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Genomic portraits of methicillin-resistant staphylococci (MRS) from food fish unveiled the genes associated with staphylococcal food poisoning (SFP), virulence and antimicrobial resistance

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Abstract

Background Characteristics of non-clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA) especially from fishery environment are poorly understood. This research, in addition to comprehensive characterisation, sought to delineate the genetic relatedness between the MRSA strains originating from clinical as well as non-clinical settings. Out of 39 methicillin-resistant staphylococcal isolates from 197 fish samples, 6 (Three each of methicillin-resistant *S. haemolyticus* (MRSH) and MRSA) with distinct resistance profiles were selected for whole-genome sequencing. Using respective bioinformatics tools, MRSA genomes were comprehensively characterized for resistome, virulomes, molecular epidemiology and phylogenetic analysis. Simultaneously, MRSH genomes were specifically examined to characterize antimicrobial resistance genes (ARGs), owing to the fact that MRSH is often recognized as a reservoir for resistance determinants.

Results Three MRSA clones identified in this study include ST672-IVd/t13599 (sequence type-SCC*mec* type/spa type), ST88-V/t2526, and ST672-IVa/t1309. Though, the isolates were phenotypically vancomycin-sensitive, five of the six genomes carried vancomycin resistance genes including the *VanT* (VanG cluster) or *VanY* (VanM cluster). Among the three MRSA, only one harbored the gene encoding Panton-Valentine Leukocidin (PVL) toxin, while staphylococcal enterotoxin (SEs) genes such as *sea* and *seb*, associated with staphylococcal food poisoning were identified in two other MRSA. Genomes of MRSH carried a composite of type V staphylococcal cassette chromosome *mec* (SCC*mec*) elements (5C2 & 5). This finding may be explained by the inversion and recombination events that may facilitate the integration of type V elements to the SCC elements of *S. aureus* with a methicillin-susceptible phenotype. Phylogenetically, MRSA from a non-clinical setting displayed a considerable relatedness to that from clinical settings.

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Conclusion This study highlights the genetic diversity and resistance profiles of MRSA and MRSH, with non-clinical MRSA showing notable relatedness to clinical strains. Future research should explore resistance gene transfer mechanisms and environmental reservoirs to better manage MRSA spread.

Keywords Antimicrobial resistance, Multilocus sequence typing, MRSA genome, ST672, ST88, SCC*mec* typing, Staphylococcal food poisoning

Background

Quality of the food is determined by various factors encompassing nutritional contents, health benefits, and importantly food hygiene. However, food safety could be compromised if proper sanitary procedures are not followed while handling, preparation and storage of food [1]. Such an ominous situation has the potential to result in foodborne diseases caused by a wide range of biological and chemical contaminants such as viruses, bacteria, parasites, chemical toxins, heavy metals, allergens, etc. [2]. Staphylococcal food poisoning (SFP), caused by staphylococcal enterotoxins, is a global common foodborne illness among various types of foodborne diseases (FBD). In the United States alone, nearly 0.24 million cases of SFP are reported every year [3]. According to a report, more than 100 individuals, including children and adults in India, exhibited symptoms of SFP and were hospitalized after consuming contaminated food. Therein, microbiological evaluation of the food revealed the occurrence of enterotoxigenic *Staphylococcus aureus*, capable of producing enterotoxins SEB and SED [4]. While the food poisoning caused by *S. aureus* remains unresolved on one hand, on the other, the pathogen is developing antibiotic resistance, creating significant challenges for control measures.

The molecular mechanisms underlying the evolution of staphylococci with adaptability towards various challenges have been a longstanding scientific paradox. To solve the mystery, plethora of studies were conducted over the years, resulting in staphylococci becoming one of the most studied pathogens. In the staphylococci, particularly coagulase-positive *S. aureus*, occasionally changing from commensal to pathogen, antimicrobial resistance (AMR) is a pressing clinical concern [5]. Virulence factors of methicillin-resistant *S. aureus* (MRSA) include the Panton-Valentine Leukocidin (PVL) toxin, which damages immune cells and contributes to severe infections, and surface proteins like Protein A, which help evade immune detection. Enzymes such as coagulase and hyaluronidase facilitate tissue invasion. These factors collectively enhance MRSA's ability to colonize, invade tissues, and resist host immune responses, leading to serious and often resistant infections [6]. Notably, public health is critical in managing MRSA due to its potential for widespread transmission and severe infections. Multidrug-resistant MRSA phenotypes complicate treatment options and heighten the need for effective infection control strategies. Besides, clonal divergence and genetic heterogeneity among the *S. aureus* that are evident from the fact that the total *S. aureus* population belonged to 10 clonal complexes (CCs) comprised of 8455 sequence types (STs) [7]. Among the varying CCs, CC1, CC5, CC8, CC15, CC22, CC30, CC45, CC93, CC97, and CC121 are 10 major ones, with CC5 having the highest number STs (1138 STs) and CC93 having the fewest, only 18. However, CC8 is the lineage of the first MRSA or archaic clone (ST250-MRSA-IV) and one of the most pathogenic clones USA300 (ST8-MRSA-IV) which constantly displays the catastrophic nature of the infection. Apart from this, 2836 STs are not even included in any of the CCs; rather exist as single, independent lineages.

At global scale dissemination of MRSA clones described the correlation between geographical location and the lineage, reflecting the varied nature of clonality from region to region [8]. For instance, USA300 clones are predominantly found in the American continent, whereas, the Bengal Bay clone (ST772-MRSA) is in Asian countries, particularly in India. This theory, however, is no longer valid because the USA300 has now been isolated from a tertiary hospital in India [9]. Similarly, other clones of MRSA, the occurrence of which was previously assumed to be restricted to specific areas, have been isolated from different regions of the world. The Bengal Bay clone of the CC1 is the sporadically detected MRSA clone in India, which has been associated with multiple cases of life-threatening infections in the Indian population [10]. In addition to the Bengal Bay clone, an ST672 MRSA clone with an immune evasion cluster distinct from ST772 was isolated from an outpatient in India with an ocular infection [11]. Apart from clinical isolation settings, the occurrence of ST672 clones was observed later from retail market fishes (non-clinical), epitomizing its transboundary spread [12]. Although clinical MRSA characteristics have been described, few research attempts have described phenotypes and genotypes from non-clinical settings such as retail market fishes, seafood products, aquaculture farms etc. [12, 13]. The genomes of non-clinical strains of MRSA ($n=3$) and methicillin-resistant *S. haemolyticus* (MRSH; $n=3$) isolated from commercial fish outlets were employed in this study to explain the distinctive features concerning ARGs, virulent determinants, and molecular epidemiology, followed by a genome comparison to shed the light on evolutionary aspects.

Methods

Bacterial isolates and the determination of their resistance profile

As a part of molecular surveillance study, 197 fish samples were collected from three major districts of Assam (Garchuk, Silagrant, and North Guwahati) using convenience sampling method. The fish samples were obtained from commercial retail markets, and they were already deceased at the time of collection. Consequently, no euthanasia procedures were necessary. To prevent contamination during transport, collected samples were brought to the lab in a sterile polythene bag under chilled condition. From the samples collected, staphylococci were isolated using the standard protocol [12]. Ten gram of fish meat (edible portion) was aseptically collected and triturated in a sterile pestle and mortar. A portion of 100 ml of sterile enrichment broth was added and the mixture was transferred to a sterile flask containing the remaining enrichment broth. The flask was then incubated further to facilitate the isolation of staphylococci. The Tryptic Soy broth (BD Difco, USA) supplemented with 1% (w/v) sodium pyruvate (Himedia, India) and 10% (w/v) sodium chloride (Himedia, India) was employed for enrichment. Presumptive staphylococcal colonies were selected using mannitol salt agar (MSA, BD Difco, USA). One hundred and ninety-seven samples yielded 39 methicillin-resistant isolates comprising of *S. aureus* ($n=23$) and *S. haemolyticus* ($n=16$), molecular identification were achieved using species-specific multiplex PCR. Of 39 isolates, six were selected based on the antibiogram and considered for whole-genome sequencing. During sampling, ethnographic information regarding the source was obtained from the local fish merchants. The isolates of this study were referred to as MRS 1 (MRSA), MRS 2 (MRSH), MRS 3 (MRSA), MRS 4 (MRSA), MRS 5 (MRSH), and MRS 6 (MRSH), and the terms were used interchangeably throughout the text.

Preliminary screening for methicillin resistance was performed using a spot inoculation test, which involved transferring 10 µl of overnight grown bacterial cultures to Muller-Hinton agar (MHA, BD Difco, USA) plates containing 6 µg/mL oxacillin (a surrogate antibiotic for methicillin) and 4% sodium chloride [14]. Cultures grown on the plate at 37 °C for 24 h incubation were considered as methicillin-resistance phenotypes. This was followed by the bacterial identification and determination of resistance pattern against 20 antibiotics that encompass 13 classes using BD Phoenix M50 instrument [15]. Multiple antibiotic resistance (MAR) index of each isolate is also calculated based on the phenotypic resistance profile [16]. Molecular identity of the isolates was confirmed by applying a multiplex PCR using the primers *23 S rRNA* (F- AGCGAGTCTGAATAGGGCGTTT; R- CCCATCA CAGCTCAGCCTTAAC) and *sodA* (F: CAAATTAAAT TCTGCAGTTGAGG; R- GGCCTCTTATAGAGACCA CATGTTA) for MRSA and MRSH, respectively, as per the cyclic conditions described elsewhere [17].

Genome sequencing

The total genome sequences (paired end) of the isolates were generated using the Illumina HiSeq 2500 platform in the MicrobesNG lab in Birmingham, UK. The FastQC tool was employed to evaluate the quality of the sequencing reads, followed by trimming the adapters with Trimmomatic. Quality check passed reads were assembled to contigs using SPAdes v 1.13.4 [18] and annotated using RAST tool kit (RASTtk) with default parameters [19]. A Quality Assessment Tool (QUAST) report on the genomes was generated to evaluate the quality of genome assembly [20]. Finally, annotated genomes were mapped to respective *S. aureus* and *S. haemolyticus* reference genomes using a progressive mauve alignment tool. The assembled genomes ($n=6$) were deposited in the Sequence Read Archive (SRA) of NCBI under the BioProject PRJNA932747 and the accession numbers SRX19312967 (MRS 1), SRX19312968 (MRS 2), SRX19312969 (MRS 3), SRX19312970 (MRS 4), SRX19312971 (MRS 5), and SRX19312972 (MRS 6).

In silico genome analysis

By using the ResFinder 4.1, and the Resistance gene identified (RGI) available in Comprehensive Antimicrobial Resistance Database (CARD), major resistance phenotypes and associated resistance genes (ARGs) were predicted [21, 22]. Similarly, virulence determinants and toxin genes were identified using VirulenceFinder 2.0 and ABRIcate-VFDB tools [23, 24]. PlasmidFinder 2.1 was employed after choosing a Gram-positive database to identify the plasmids in the genome [25]. The presence of mobile genetics elements (MGE) was identified with the assistance of Mobile Element Finder v1.0.3 [26]. The genes involved in the secondary metabolite biosynthetic pathway and the production of bacteriocin were identified by using antiSMASH 5.0 [27]. Molecular epidemiology of the isolates was recognized employing staphylococcal cassette chromosome (SCC) *mec*-typing, *spa*-typing (*S. aureus* ($n=3$) only), and multilocus sequence typing (MLST; *S. aureus*) that used SCCmecFinder 1.2 [28], spaTyper 1.0 [29], and MLST 2.0 [30], respectively. The accessory gene regulators (*agr*) types of three MRSA genomes were determined using appropriate primers [31] by an In-silico PCR method [32].

Genome comparison and phylogeny

For the genome-wide comparison of two ST672 MRSA clones (MRS 1 and MRS 4) following clinical ST672 strains were used; ST672-IVd (JAJTJT000000000, [33]), AMRF2 (JASM00000000, [34]), GR1 (AJLX00000000,

[35]), VB12268 (LXWS00000000, [36]), and VBV169 (LWMG00000000, [36]). According to the corresponding reference literature, ST672-IVd, AMRF2, GR1, VB12688, and VBV169 clones were isolated from a male patient (tertiary health care center, Kerala), ocular infection, blood septicemia, skin and soft tissue infection (SSTI), and sepsis, respectively. Similarly, MRS 3 (ST88) was compared with 15 previously reported ST88 MRSA strains. Detailed information on the source, country and year of isolation, etc. of reference genomes provided in Supplementary material 1. A genome-based phylogenetic tree was constructed using the Type/Strain Genome Sever (TYGS) with the genome of *Escherichia coli* as outgroup [37]. The tree was annotated using the interactive Tree of Life (iTOL) v6.8 [38]. The average nucleotide identity (ANI) was calculated based on Blast+for the ST672 and ST88 MRSA genome of this study against the respective reference genomes using the JSpeciesWS webserver [39].

Results

Ethnographic research

In this study, fish samples from which the isolates have been identified were either river-caught or intra country imported from other states namely Andhra Pradesh or Kolkata of India. Detailed information on the sample sources is shown in Table 1. Sampling area was selected based on the ethnographic information that the chosen sites were within the proximity of hospitals and factories. According to the local residents, the rural waste management system is poorly regulated in Assam. Therefore, it is assumed that medical wastes and industrial effluents are discharged directly to the vicinities of aquatic resources. This may eventually lead to the selection of AMR bacterial pathogens, which could be one of the main reasons why water resources are the epicentre for the propagation of AMR.

Bacterial strains and their phenotypic resistance

The BD Phoenix instrument identified three of the six isolates as *S. aureus* and the remaining three as *S. haemolyticus*. The multiplex PCR yielded the amplicons of 894 bp ($n=3$) and 531 bp ($n=3$) which corresponds to *S. aureus* and *S. haemolyticus*, respectively. Furthermore, resistance patterns revealed that all six isolates (100%) have multidrug-resistance (MDR) phenotype which means, they were resistant to at least one antibiotic from three or more classes. Notably, MRSA ($n=3$) showed almost identical resistance patterns regardless of the source of isolation, except for MRS 1 which exhibited additional resistance to trimethoprim/sulfamethoxazole (SXT) antibiotic. As a result, MRS 1 had the highest MAR index (0.4), while the other five isolates had 0.35. The resistance profile of individual isolates is given in Table 2.

Resistance and virulence genotypes

The circular map of two representative genomes is shown in Fig. 1, and the remaining circular maps are depicted in Supplementary Material 2. Genomic features of six staphylococcal isolates are provided in Table 3. A representative mauve alignment of the MRS 1 genome against the *S. aureus* reference genome is illustrated in Fig. 2. Screening for resistance and virulence determinants revealed that all three MRSA isolates were equipped with an extensive array of ARGs and toxin genes, while MRSH isolates carried a handful of ARGs. Based on the distribution of resistance genes, six genomes were divided into two clades namely clade1 (comprising of three MRSA genomes) and clade 2 (including three MRSH genomes) (Fig. 3a). Even though all the isolates were phenotypically

Table 1 Details of sources of sampling

SL No	Isolate ID	Sample source		Organism identified	Location	Latitude and Longitude	Imported/Farm-raised/ River-caught[#]
		Local name*	Scientific name				
1	**MRS 1**	Bhangan	*Labeo boga*	*Staphylococcus aureus*	Gauripur, Silagrant	26°13′19.7″ N: 91°41′50.9″ E	Brought from Ghograpar, Nalbari District)
2	**MRS 2**	Catla	*Catla catla*	*S. haemolyticus*	Jaiguru Market, Amingaon, Silagrant	26°12′31.7″ N: 91°41′8.5″ E	Brought from Rangiya, Kamrup District
3	**MRS 3**	Kuri mas	*Labeo gonius*	*S. aureus*	Near Ayursundra Hospital, Garchuk	26°6′25.3″ N: 91°43′11.2″ E	River-caught (from a lake in Hajo villiage)
4	**MRS 4**	Bhangan	*Labeo boga*	*S. aureus*	Near Ayursundra Hospital, Garchuk	26°6′25.3″ N: 91°43′11.2″ E	River-caught (from a lake in Hajo villiage)
5	**MRS 5**	Rupchanda	*Pygocentrus nattereri*	*S. haemolyticus*	Gauripur, Silagrant	26°13′2.3″ N: 91°42′6.3″ E	Imported from Kolkata
6	**MRS 6**	Shrimp	*Penaeus sp.*	*S. haemolyticus*	IIT Guwahati Campus fish market, Silagrant	26°11′42.7″ N: 91°41′17.2″ E	Imported from Andhra Pradesh

*Local name is collected from the fish merchants

[#] According to the information provided by the fish vendors

Table 2 Bacterial isolates and their resistance phenotypes

SL No	Isolate ID	Organism	Resistance profile*	MAR index = A/B#	Secondary metabolites
1	MRS 1	*Staphylococcus aureus*	AMP-CFZ-FOX-GEN-NOR-OXA-PEN-SXT	8/20 = 0.4	aureusimine, staphyloferrin A
2	MRS 2	*S. haemolyticus*	AMP-CFZ-FOX-GEN-OXA-PEN-TET	7/20 = 0.35	staphyloferrin A
3	MRS 3	*S. aureus*	AMP-CFZ-FOX-GEN-NOR—OXA-PEN	7/20 = 0.35	aureusimine, staphyloferrin A
4	MRS 4	*S. aureus*	AMP-CFZ-FOX-GEN-NOR-OXA-PEN	7/20 = 0.35	aureusimine, staphyloferrin A
5	MRS 5	*S. haemolyticus*	AMP-CFZ-FOX-GEN-OXA-PEN-SXT	7/20 = 0.35	staphyloferrin A
6	MRS 6	*S. haemolyticus*	AMP-CFZ-FOX-ERY-GEN-OXA-PEN	7/20 = 0.35	staphyloferrin A

* Antibiotics and abbreviations: AMP-ampicillin; CFZ-cefazolin; FOX-cefoxitin; ERY-erythromycin; GEN-gentamicin; NOR-norfloxacin; OXA-oxacillin; PEN-penicillin; SXT-trimethoprim-/sulfamethoxazole; TET-tetryacycline

Multiple antibiotic resistance (MAR); 'A' is the number of antibiotics to which isolates were resistant; 'B' is the total number of antibiotics to which isolates were exposed

sensitive to vancomycin, all the isolates except MRS 2 harboured either *VanT* gene in the *VanG* cluster or the *VanY* gene in the *VanM* cluster which usually confer resistance to glycopeptides. In addition to the ARGs, genes encoding efflux machinery (*sepA*) that pumps out the antiseptics and disinfectants of the cell are also identified in most of the isolates. The incompatibility plasmid Inc18 (rep16 family) and Rep3 (rep5a family) were the plasmids found in all the MRSA isolates with ISSau5 as the commonly found MGE. In the case of MRSH, RepL (rep10 family) was found in both MRS 5 and MRS 6, while MRS 5 and MRS 6 additionally carried RepA_N (rep39 family) and Rep-Trans (rep7a family), respectively. All the MRSH isolates carried *ISSha1* while MRS 5 and MRS 6 further carried *IS256*.

When it comes to virulence determinants, all the three MRSA isolates carried an extensive battery of virulence factors, whereas the coagulase-negative MRSH yielded no results during genome analysis. Among the three MRSA, one isolate (MRS 3) carried gene coding for Panton-Valentine Leukocidin toxin (PVL) i.e., *lukS-lukF PV* locus, while the other two carried only *lukF* component. Identification of *icaADBC* operon along with certain other biofilm-associated genes such as *clfA*, *fnbpA*, *fnbpB*, etc. in all three MRSA isolates justified the PIA-dependent biofilm-production. Virulence determinants identified in each MRSA genome are showed in Fig. 3b. The antiSMASH screening revealed the presence of the secondary metabolite aureusimine in three MRSA isolates, while the staphyloferrin A locus was found in all six genomes, including MRSA and MRSH (Table 1). Similarly, staphylobactin was found in all three MRSA isolates, but the sequence similarity was only 87%.

Molecular epidemiology of MRSA and MRSH isolates

Of three MRSA, two isolates (MRS 1 & MRS 4) belonged to the same ST i.e., ST672 with an allelic profile of *arcC*-4; *aroE*-3; *glpF*-1; *gmk*-1; *pta*-11; *tpi*-72; *yqiL*-11 but, two different *spa* types such as t13599 (MRS 1; repeat succession: 26-22-17-16-16) and t1309 (MRS 4; repeat succession: 26-22-17-20-17-12-17-17-16-16). Whereas the remaining MRSA belonged to sequence type ST88 (22-1-14-23-12-4-31), and *spa* type t2526 (7-12-21-17-13-13-13-34-33-13). Similarly, MRS 2 and MRS 6 *S. haemolyticus* isolates belonged to ST8 with allelic profile of RiboseABC-4; SH1431-2; SH1200-5; arcC-1; cfx-1; hemH-1, leuB-1 while the MRS 5 *S. haemolyticus* belonged to ST21 (4-5-5-1-1-6-1). Of three MRSA, MRS 1 (ST672-MRSA-t13599) carried type IVd (2B) SCC*mec* elements whereas, MRS 3 (ST88-MRSA-t2526) and MRS 4 (ST672-MRSA-t1309) carried type IVb (2B) and type V (5C2), respectively. Besides, all three MRSH isolates had the composite type V (5C2 & 5) SCC*mec* elements. According to *agr* typing of MRSA isolates, MRS 1 and MRS 4 belonged to *agr* type I, while MRS 3 to *agr* type III.

Genetic relatedness among the MRSA strains

Results from the ANI (based on blast) revealed that the three MRSA strains used in this study had the highest level of resemblance to the respective ST672 and ST88 reference groups. The MRS 3 (ST88) strain shared the highest nucleotide identity of 99.88% with the HST-105 strain isolated from the human sample (Supplementary material 3). Similar results were obtained by MRS 1 and MRS 4, which both displayed 99.96% identity with VBV169 (isolated from sepsis) and 99.92% similarity to the reference genome ST672-IVd (isolated from a pus sample), respectively. The circular comparison between the query and reference genomes, accomplished by BRIG analysis supported the ANI findings that the genome of this study demonstrated a high level of similarity with the clinical MRSA strains which were used as the reference genomes (Fig. 4a and b). The gaps in the concentric rings of the BRIG image denote the sequences present in the reference but missing in the query, indicating the draft nature of the genome of this study. Based on the phylogenetic analysis, two MRSA clades namely the ST672 clade and ST88 clade, and a singleton (ST672/t13599) were identified (Fig. 5). The ST88 clade encompassed all the MRSA that belonged to ST88 and the ST672 clade comprised all ST672 MRSA except MRS 1 which existed as a singleton.

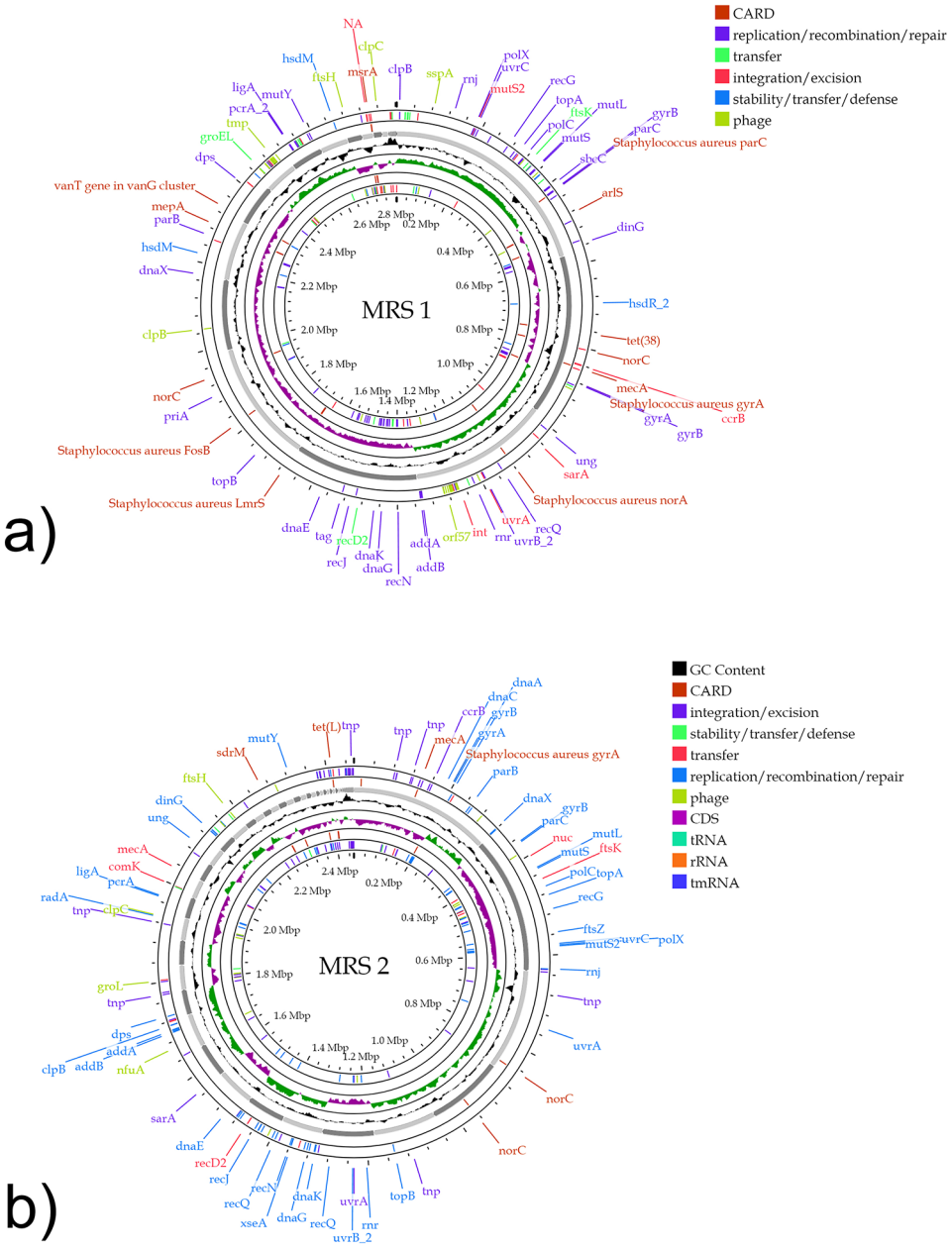

Fig. 1 Circular map of representative methicillin-resistant*Staphylococcus aureus*(MRSA): (**a**) and methicillin-resistant *S. haemolyticus* (MRSH) (**b**) genomes highlighting the major resistance genes and mobile genetic elements (MGE)

Discussions

MRSA is an unpredictable pathogen that undergoes several episodes of genomic rearrangements to improve its adaptability and survival. Owing to the inappropriate use of antibiotics, the prevalence of MRSA in hospitals and healthcare settings is not uncommon. However, special consideration should be given to its occurrence in the fishery environment, which is a non-clinical setting. Evidence from previous studies mounting that, multiple entrances is available for the AMR pathogens to infiltrate into the fishery environment [40]. The rationale behind isolating and characterizing MRSA from fishery environment lies in the potential implications for public health and the understanding of antibiotic resistance in diverse environments. Fishery environments can serve as reservoirs for various bacteria, including MRSA, due to the use of antibiotics in aquaculture practices. Similarly, MRSA is a known human pathogen, and its presence in retail market fishes raises concerns about the potential transmission to humans through direct contact or consumption of contaminated seafood. Understanding the prevalence and characteristics of MRSA in aquaculture

Table 3 Genomic features of Staphylococci

	MRS 1	MRS 2	MRS 3	MRS 4	MRS 5	MRS 6
# contigs (>=0 bp)	68	115	100	179	464	39
# contigs (>=1000 bp)	17	44	22	56	67	13
Total length (>=0 bp)	2,803,340	2,455,896	2,836,797	2,535,664	2,672,354	2,800,965
Total length (>=1000 bp)	2,787,016	2,435,085	2,818,396	2,499,296	2,497,610	2,793,338
# contigs	22	52	25	67	159	15
Largest contig	572,671	322,121	1,325,285	271,310	222,407	1,050,538
Total length	2,790,446	2,440,623	2,820,448	2,506,864	2,556,093	2,795,165
GC (%)	32.72	32.71	32.64	32.73	33.04	32.71
N50	318,982	117,454	278,962	78,337	65,019	564,525
N90	85,303	25,325	92,366	22,038	17,293	123,253
auN	332289.1	156388.4	720852.2	109077.4	87579.2	608026.4
L50	4	6	2	10	11	2
L90	9	22	8	31	40	6
# N's per 100 Kbp	0.00	0.00	0.00	0.00	0.00	0.00

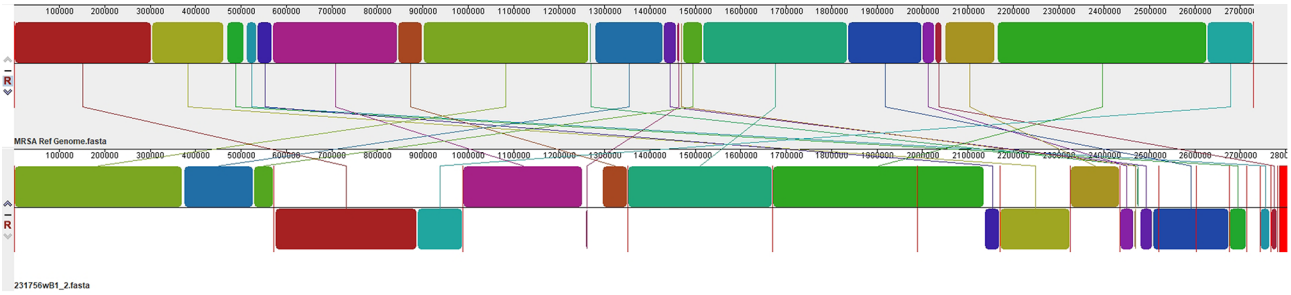

Fig. 2 Progressive mauve alignment: Genome mapping of methicillin-resistant *Staphylococcus aureus* (MRSA) of this study against the reference genome using progressive mauve alignment

can aid in assessing the associated health risks. According to information provided by local vendors and fish merchants, for significant percentage of the Assamese population fish constitutes one of the important food items. It is also true that despite the high cost of fish, the Assamese are very particular about including fish in their quotidian consumption. They consume not only river-caught (indigenous) fishes but, a wide variety of fishes of intra-country imports originating from different marine coastal states, in addition to the farm-raised fishes being cultured in neighboring areas of Assam.

One of the advantages of globalization is that trans-boundary transportation of men and materials that include fish-based food stuffs wherein the contents sourced from different nations in shortest periods of time [41]. The flip side is facilitation of mobility of AMR containing microbes with same pace are posing serious threat to wellbeing not just animals, plants and humans but the environment too is seeking refuge in approach of "One Health" as ultimate remedy. Out of a total of 193 countries in the world 77% (148) of them share two rivers, 15% (30) three rivers 0.047% (9) through four and 0.067% (13) through five or more countries. Contaminated waters, untreated effluents in the form of rivers transmit number of diseases is serious negative externality the countries must deal with [42]. In the absence of proper containment procedures that are pragmatic in nature the economic loss due to microbial infections from the environment and foods coupled with antimicrobial resistance is enormous [43].

Analogous to the health care settings, imprudent use of antibiotics becomes the leading cause of AMR in the fisheries. Antibiotics when mixed with fish feeds, the indigestible and/or unabsorbed antibiotics can reach the water bodies through faeces and their prolonged persistence can exert a selection pressure of the antibiotics [44]. When pathogens develop resistance, it is much easier to transfer resistance determinants because most of the acquired ARGs in bacteria are carried on mobile genetic elements. When it comes to commercial fish outlets, hygienic practices to be followed by the fish handlers play a crucial part in the spread of AMR. In this study, MRSA and MRSH isolates are identified from local fish markets of Assam, and this is inviting to assume that the fish samples are contaminated by either improper fish handling or water and ice that are used for their preservation. A similar study by Sivaraman and his team reported the occurrence of MRSA in retail market fishes and processed seafood products from Veraval, India [45]. According to their findings, it is crucial to practice impeccable hygiene

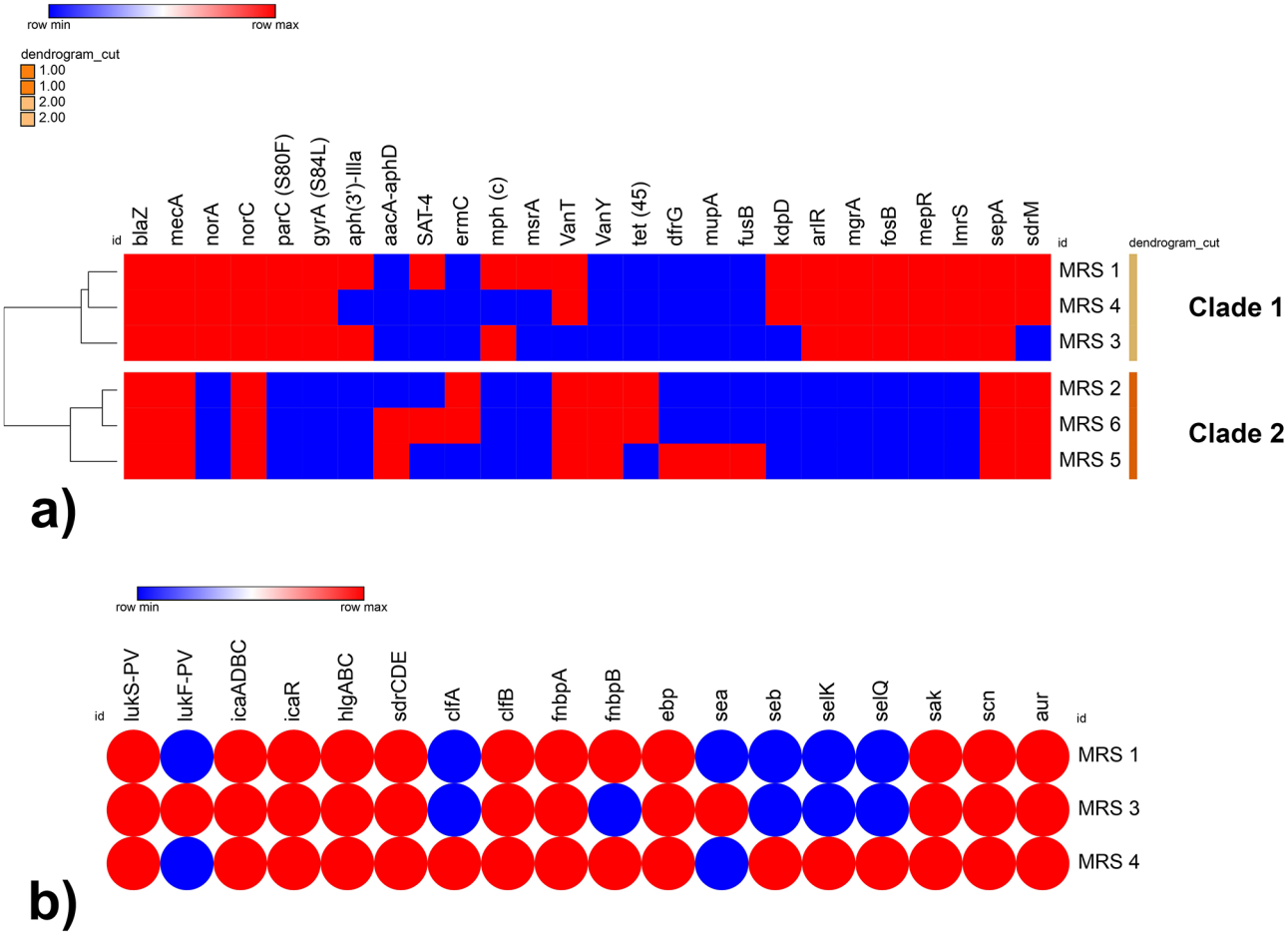

Fig. 3 Distribution of major antimicrobial resistance genes (ARGs) virulence determinants: **a**: Identification of ARGs and clustering of methicillin-resistant staphylococci ($n=6$) based on the presence and absence of ARGs. **b**: Virulence determinants distributed among three methicillin-resistant *Staphylococcus aureus* (MRSA) strains. In both the figures, red colour indicates the presence of genes whereas blue denotes the absence

when handling fish to prevent contamination, which perfectly align with findings of this study that following good fish-handling practices throughout the supply chain is indispensable.

The presence of ARGs in the isolates was consistent with their phenotypic resistance profiles, with the exception that all the isolates were sensitive to vancomycin despite the presence of VanT/VanY genes. Since the percentage identity of VanT/VantY matching regions was lower than usual, this finding was given less significance. Besides, our findings recorded that the expression of efflux pump encoding genes (*arlS*, *norC*, *norA*, etc.) can confer resistance to fluoroquinolones. Aside from the *aacA-aphD* locus, which confers resistance to aminoglycosides by modifying the antibiotics, *kdpD* genes found in MRSA genomes also confer resistance through the efflux mechanism. The relation between mobile genetic elements and ARG transfer is well established. In this study also, it has been noted that all six genomes carried at least one MGE. When the insertion sequence (IS) was examined, *ISSau5* was the MGE found in MRSA isolates, while *ISSha1* and *IS256* were in MRSH isolates. Previously, *IS256* was identified in nosocomial *S. epidermidis* isolates and the study specifically discussed the association of *IS256* with aminoglycoside resistance and biofilm formation [46]. Similarly, *ISSha1* is typically identified in *S. haemolyticus*. However, Li et al. (2011) observed that the *ISSha1* was integrated into J1 (junk region) of type X SCC*mec* elements of the *S. aureus genome*, which is speculated to have been transferred from *S. haemolyticus*, which highlights the importance of insertion sequences in gene transfer and acquisition [47]. In this context, ISs are omnipresent in prokaryotes which take part in genome evolution. Despite being small and genetically compact, ISs form a part of transposon yet, do not encode any functional proteins other than those involved in the gene transference. Notably, studies suggested that several ISs are clustered within the plasmid genomes in a region called 'islands' and play a pivotal role in plasmid integration and excision [48].

S. aureus can produce a plethora of virulence factors such as enterotoxins, exfoliative toxins, leukotoxins,

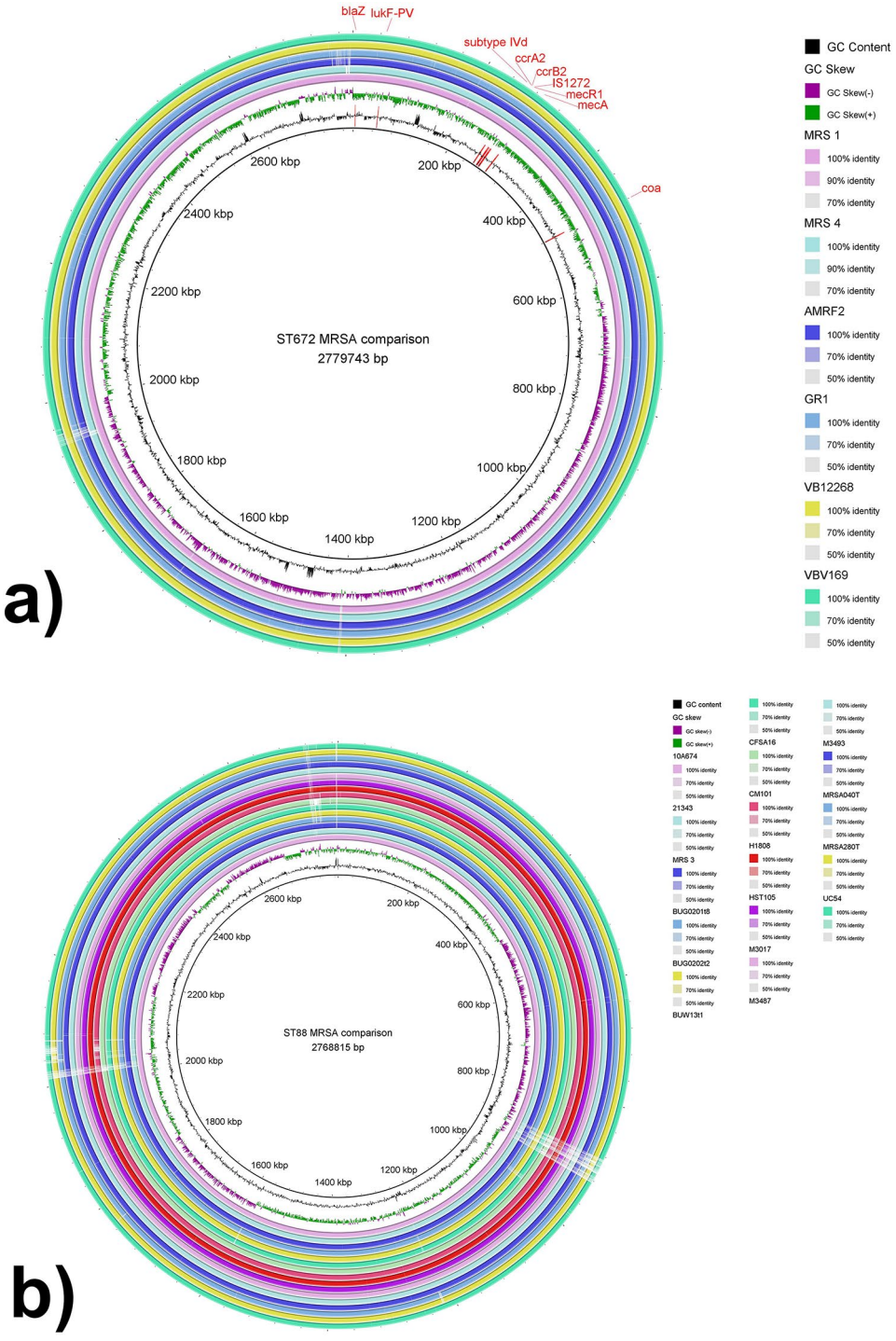

Fig. 4 Blast Ring Image Generator (BRIG) analysis of the genomes of this study against the clinical reference genomes: **a**: BRIG output of ST672 MRSA; **b**: BRIG output of ST88 MRSA. The gaps (white area) in the concentric ring represent the absence of genes in query sequences which are present in reference genome (draft nature)

hemolysin, etc. However, gene-level expression of these toxins is regulated by an accessory gene regulator (*agr*) system with the help of a communication molecule namely autoinducing peptides (AIP) [49]. Staphylococcal enterotoxins (SEs) are bifunctional toxins having the role of gastrointestinal toxins and super antigens that are structurally located in two different domains [50]. Among the several classical-SEs and SE-like toxins reported so

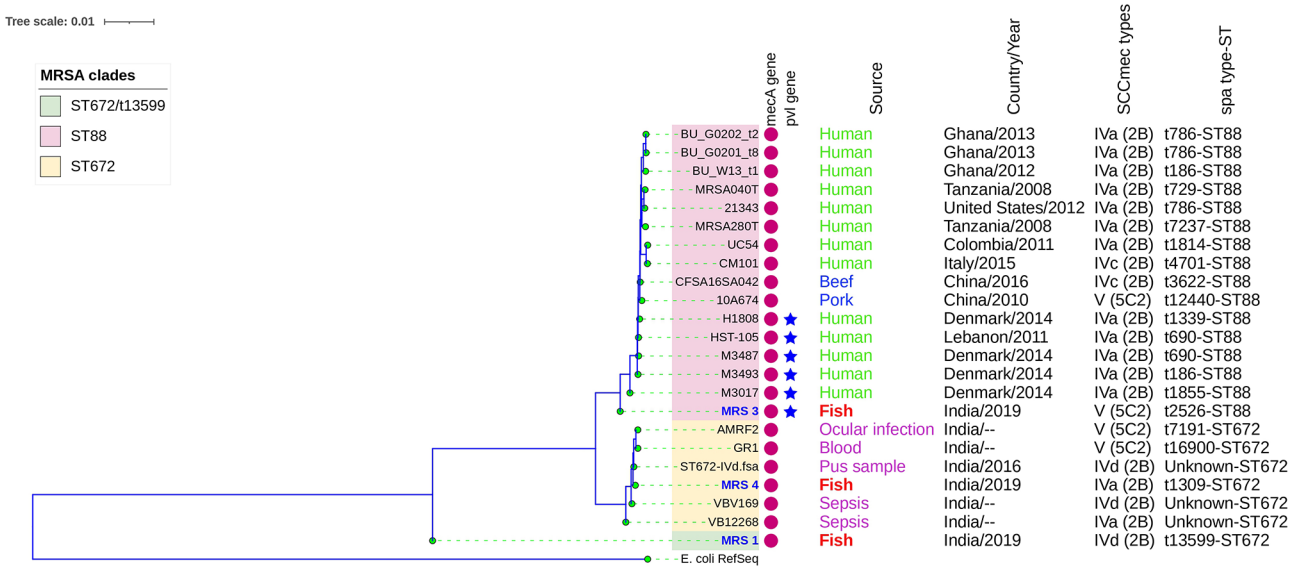

Fig. 5 Phylogeny of clinical and non-clinical MRSA strains: Genetic relatedness among the methicillin-resistant *Staphylococcus aureus* (MRSA) isolates of the present study and selected clinical reference genomes. Label with blue bold characters are the genomes of the present study, isolated from fish samples

far, staphylococcal enterotoxin A (SEA) and SEB are the major classical-SEs, and SEG and SEI are the two SE-like SEs identified in the current study. Previous studies have illustrated the SEA as the most common toxin associated with food poisoning whereas the SEG and SEI also play a minor role in foodborne illnesses [51]. Importantly, the strains used in this study were isolated from retail market fishes, and they can produce toxins having a significant role in foodborne illness. It is noteworthy that SEs are significantly resistant to heat (heat stable) and may hold some of their biological functions even after pasteurization, which uses extremely high temperatures (121 °C for 28 min) [52]. In this case, question remains whether the consumption of fishes, though properly cooked, however, is contaminated with enterotoxins will pose health hazard? Accumulating evidence proved that inappropriate refrigeration and inadequate pasteurization are the two major drivers of staphylococcal food poisoning, the second most frequently encountered food-borne illness [53]. Biofilm producing capacity of *S. aureus* represents yet another virulence property. The *agr* system governs the transition from planktonic to biofilm lifestyle in the same manner that it regulates toxins gene expression. Even though biofilm is beneficial in dealing with various hostile conditions, there is an interplay between biofilm production and antimicrobial resistance. A study has proved that the penetration of certain antibiotics such as oxacillin, cefotaxime, and vancomycin has been reduced by the *S. aureus* and *S. epidermidis* biofilm [54]. This implies that, in addition to the various resistance genes identified, biofilm production by the MRSA isolates of this study may also contribute to reduced susceptibility to conventional antibiotics. Another distinguishing feature of biofilm development is the progression of infection in the host, in which the bacterial cells detach from the biofilm and migrate to uninfected areas of the host to initiate a nascent biofilm formation, resembling the migration of metastatic cancer cells [55]. As a result, the identification of biofilm-producing *S. aureus* in non-clinical, retail market fish samples call for increased molecular surveillance.

Understanding the molecular epidemiology of MRSA may define the risk elements associated with its infections and assist to design successful therapeutic approaches. Among the various bacterial typing methods, *agr* typing, *spa* typing, MLST, and SCC*mec* typing offer advantages for distinguishing MRSA, with SCC*mec* typing also applicable to coagulase-negative staphylococci (CoNS). Previously, *S. haemolyticus* belonging to ST8 and ST21 have been reported from various infections and clinical settings [56, 57]. However, our findings were paramount since the ST8 and ST21 MRSH were identified from a non-clinical setting i.e., commercial fish outlet. The current study is therefore more likely to be the first to report these two MRSH clones from an environment beyond clinical and infection cases. Although only six genomes were examined, our findings indicated that the number of clones was higher, particularly in the case of MRSA where three isolates were distinct from each other. Earlier research identified the ST672 MRSA as an emerging disease clone predominantly found in India [11, 58]. On the contrary, a high prevalence of ST672 MRSA was observed in Kuwait hospitals [59]. In this context, the contribution of international travel to the global spread of MRSA cannot be overstated. A comprehensive review

of the globalization of MRSA has documented the occurrence of ST772 MRSA (also known as Bengal Bay clone) which was assumed to be prevalent in Asia, in various countries such as Germany, United Kingdom, and Malaysia through international travel [60]. Similarly, owing to its high prevalence in African countries, ST88 MRSA was previously considered to be established as an 'African' community-associated (CA)-MRSA clone [61], but has now been identified in India and China recently [12, 62]. Once introduced to a country, MRSA spread within the community is highly facilitated by various factors majorly direct contact with patients or objects. Apart from this, transference of methicillin resistance from resistant to susceptible staphylococci plays a pivotal role in the local and transboundary transmission of MRSA and it is greatly influenced by the occurrence of SCC*mec*. MRSA in this study belonged to either type V or type IV, which were previously thought to be CA-MRSA markers, but subsequent studies discovered overlapping of typical characteristics, indicating that such classification of MRSA is not significant. Conversely, our findings of MRSH genomes carrying the composite type V SCC*mec* elements (5C2 & 5) coincided with a previous finding wherein the ST672 MRSA belonged to the same category [35]. Perhaps, the integration of type V elements to already existing SCC*mec* in methicillin-susceptible *S. aureus* (MSSA) could be a possible reason for the presence of the composite elements. The question of how closely genetically related MRSA isolates from non-clinical settings are to those from clinical settings was one of the main goals of creating a phylogenetic tree. To address this, in addition to the three MRSA genomes examined, this study also included 20 reference genomes, including ST88 ($n=15$) and ST672 ($n=5$) isolated from clinical settings. Our findings demonstrated that all the MRSA clones from this study displayed considerable homology with the reference genomes, regardless of country and source of isolation (Fig. 4). However, MRS 1 was distinct from other clinical ST672 MRSA strains even though it belonged to the same ST672 subgroup. Besides, MRS 3 was closely related to the Lebanon and Denmark MRSA strains with all of them harbouring the *pvl* gene. Even though only two STs were identified in this study, most of the genomes were genetically diverged based on the *spa* types, 15 different spa types were identified among 23 genomes (including references).

Conclusions

In summary, isolation and characterization of MRSA from commercial fish outlets is motivated by the need to assess the potential hazards to human health, monitor antibiotic resistance patterns, adopt a One Health perspective, investigate environmental reservoirs, and understand the ecological implications. This research contributes to the broader efforts to mitigate the spread of antibiotic resistance and promote the sustainable and safe practices in aquaculture. While the ST88 and ST672 MRSA strains have been reported previously in clinical settings, their occurrence in non-clinical setting such as retail market fish raises serious concerns. Our description of these two MRSA strains shed light on the genomic portraits of emergence and adaptation in the non-clinical settings. Since the MRSA isolates of this study carried genes (*sea*/*seb*) encoding thermo-stable staphylococcal enterotoxins, risk of MRSA as the food-borne pathogens are dangerously high. Besides, the genetic similarity between MRSA from clinical and non-clinical settings were prompting to hypothesize that these two lineages descended from a single ancestor, highlighting the transboundary spread of MRSA. While reporting the occurrence and characteristics of MRSA in commercial fish outlets, the present findings emphasized the urgent need for a large-scale surveillance encompassing a range of settings from humans to animals, food, environment, and other related sectors, etc.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

MH: Conceptualization, data curation, formal analysis, investigation, methodology, software, writing original draft. GKS: Conceptualization, funding acquisition, project administration. MPM: Conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, writing-review and editing.All the authors critically reviewed, modified and approved the final version of the manuscript.

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Data availability

The genome sequences of the isolates used in this study are available at SRA-NCBI under the BioProject PRJNA932747 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA932747).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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