Title: Prevalence of virulent and biofilm forming ST88-IV-t2526 methicillin-resistant *Staphylococcus aureus* clones circulating in local retail fish markets in Assam, India

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Abstract

The burden of antimicrobial resistance (AMR), particularly in India has been increased alarmingly. Methicillin-resistance in *Staphylococcus aureus* has been recognized as serious threat to the human especially if they are biofilm forming and equipped with virulence factors. In the present study monitoring of antibiotic resistant *S. aureus* was performed at three selected sites in Assam, India in August 2019 and February 2020. Ethnographic information was collected from the fish vendors in order to track and address potential sources of contamination. Twenty three potential methicillin-resistant *S. aureus* (MRSA) strains were identified from fish sold by these vendors and subjected to molecular characterization. The antimicrobial resistance profile of these MRSA strains were regarded as multidrug-resistant (MDR) as they were resistant to ≥3 classes of antibiotics. The most prevalent resistance profile was; ampicillin-cefazolin-cefoxitin-gentamicin-norfloxacin-oxacillin-penicillin. Accessory gene regulators III (*agr* III) type MRSA (18/23, 78.26%) were found to be predominant compared to *agr* I type (5/23, 21.74%). Four isolates (17.39%) were observed to carry SCC*mec*-IV elements, which is a typical feature of community-associated MRSA (CA-MRSA). Two SCC*mec*-IV MRSA isolates were found to harbour panton-valentine-leucocidin (PVL) toxin genes and were resistant to macrolide in addition to beta-lactams. MLST and *spa* typing identified all MRSA as ST88 with *spa* type t2526. This is the first report from India on the incidence of ST88-SCC*mec*-IV (ST88-IV) MRSA in a fish market and its aquatic environs. The high prevalence of a single MLST clone, ST88, suggests that this lineage has a unique survival advantage in this environment. The study discusses the contribution of hospital wastewater in the dissemination of pathogenic MRSA clones to aquatic resources and then to humans through the food chain.

Keywords: *CA-MRSA*, *Molecular epidemiology*, *Virulence genes*, *biofilm-associated genes*, *Fish samples*

1. Introduction

Staphylococcus aureus is an important opportunist pathogen found on the skin and in the nasal cavity of people and animals. It can cause a wide range of diseases and toxinoses. *S. aureus* may be responsible for life threatening disease such as bacteraemia or sepsis, and can lead to pneumonia in people with underlying pulmonary disorders. Deep-seated infections such as osteomyelitis and endocarditis are also attributed to *S. aureus* (Rehm et al., 2008). Additionally, *S. aureus* is equipped with an extensive array of virulence factors associated with disease and food poisoning such as enterotoxins, exfoliative toxins, toxic shock syndrome toxin-1, Panton-Valentine Leucocidin (*pvl*), staphylococcal complement inhibitor and haemolysin. Staphylococcal scarlet fever (SSF) and toxic shock syndrome (TSS) are two rare superantigen mediated infections that can result in non-specific, excessive stimulation of T-cells. Antimicrobial-resistance (AMR) in *S. aureus* has been recognised as a global threat, as resistance to the drugs constrains therapeutic opportunities (Foster, 2017). The history of methicillin-resistance in *S. aureus* dated back to 1960 when the first case of methicillin-resistant *S. aureus* (MRSA) was reported in United Kingdom, two years after the discovery of methicillin. Since then, the incidence of MRSA, particularly in India has escalated alarmingly and is still on the rise. For instance, the numbers of MRSA isolated increased from 29% to 47% during 2009 to 2014 (Kulkarni et al., 2019). Owing to the new antibiotic paradox, India has witnessed a considerable rise in AMR-attributed mortality and morbidity. The transition of methicillin-susceptible *S. aureus* (MSSA) to MRSA results from the acquisition of a methicillin-resistance determinant; the *mecA* gene found on a highly transmissible genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Sekizuka et al., 2019). A homologue of *mecA* called *mecC* was first reported in 2011 (García-Álvarez et al., 2011) and is found in MRSA which are differentially resistant to cephalosporin (Kim et al., 2012). Initially, MRSA was found to be a nosocomial pathogen, associated predominantly with hospital and nursing home infections. Subsequently, a number of MRSA infections were observed in patients without exposure to healthcare institutions caused by different MRSA lineages now referred to as community-associated MRSA (CA-MRSA). Clinical

manifestations of CA-MRSA include mild skin and soft tissue infections and also fatal necrotizing pneumonia. CA-MRSA lineages include ST1-IV (USA400), ST8-IV (USA300), ST80-IV (European clone) and ST30-IV (Southwest pacific clone). Although initially found in North America numerous cases of infection have been reported from different parts of the world (Kong et al., 2016). CA-MRSA lineages typically have smaller SCC*mec* types and often possess a gene producing PVL.

Aquaculture plays a crucial role not only in delivering nutrition but also through its economic contribution. In Assam, India the fishery sector provides around 2% of gross state domestic product (GSDP) to the state economy every year (Gogoi et al., 2015). Approximately 0.3 million hectares of land in Assam is covered with a variety of water resources including ponds, rivers and beels (lake-like wetlands). Environmental conditions in this state are congenial for freshwater fish cultures for species native to sub-tropical climates and there is significant piscine diversity in the local ecosystem. In spite of advantages, the state has not still achieved self-sufficiency to meet the fish demand from the population in Assam owing to insufficient local fishing or aquaculture industry, unsustainable utilization of local aquatic resources and inappropriate farming system approaches (FSA). However, better awareness of fish farming approaches, scientific farming practices, and accessibility to new farming technologies among local farmers is expected to bring progressive growth in aquaculture. Recently, the studies from India have attested the incidence of MRSA in foods of animal origin and aquaculture settings (Bhowmik et al, 2021; Sannat et al 2021). The purpose of the present study was isolation of MRSA and its molecular characterizations of toxic and biofilm-associated genes in order to understand the molecular epidemiology of the MRSA from fish environs.

2. Methods

2.1 Sample collection

An ethnographic study was performed prior to sampling based on which the study sites were selected. Accordingly, samples were collected in August, 2019 and February, 2020 from retail markets and natural water bodies located in three distinct

sites: Silagrant (site 1, 26.176570° N, 91.689732° E), Garchuk (site 2, 26.104791° N, 91.712418° E) and North Guwahati Township Committee (site 3, 26.193736° N, 91.721780° E). The three study sites were characterised by the presence of natural water bodies having proximity to the hospitals and various industries. This confounded at the possibility of water bodies getting contaminated by these hospital and industrial effluents. In view of this, a total of 173 samples comprising diverse piscine fauna were collected from site 1 (n=45), site 2 (n=54) and site 3 (n=74). Samples were immediately transferred to a sterile polythene bag on ice and transported to the laboratory in a chilled condition. The fish samples were macerated and aseptically transferred to the staphylococcal enrichment media (tryptic soy broth supplemented with 1% sodium pyruvate and 10% sodium chloride). The mannitol salt agar (MSA) was inoculated with enriched culture and the typical yellow colony (mannitol-fermenting) were picked and subjected to further studies.

2.2 Identification of S. aureus and its antimicrobial susceptibility test (AST)

The necessary precautions were taken and the biosafety practices were strictly followed for handling and disposal of the bacterial cultures and other hazardous chemicals. The antibiotic susceptibility of the confirmed MRSA isolates was determined using different beta-lactam (cephalosporin, cephamycin and penicillins) and non-beta-lactam (fluroquinolones, aminoglycosides, rifamycin, glycopeptides, folate pathway antagonists, lincosamides, lipopeptides, macrolides, nitro heterocyclics, oxazolidinones, streptogramin and tetraycyclins) antibiotics. The *S. aureus* cultures were enriched and then isolated on mannitol salt agar (MSA). BD Phoenix™ M50 system (BD Diagnostic Systems, Sparks, MD) was employed for the bacterial identification and to study AST profile of the selected isolates (Hong et al., 2019). Clinical and Laboratory Standard Institute (CLSI) guidelines were followed for the interpretation of the resistance patterns (CLSI, 2020). Multiple antibiotic resistance (MAR) index was calculated for each isolates as the ratio of number of antibiotics to which isolates showed resistance to the total number antibiotics to which isolates were exposed (Sivaraman et al., 2020).

2.3 Phenotypic and genotypic confirmation of MRSA

Mannitol-fermenting colonies (n=5) with the typical appearance of *S. aureus* were picked from each mannitol salt agar (MSA) plate to screen the phenotypic resistance to oxacillin (CLSI, 2020). The cell suspension were prepared by inoculating Brain Heart Infusion (BHI) broth with presumptive S. aureus colonies which was then spot inoculated on Mueller-Hinton Agar (MHA) containing oxacillin (6µg/mL) and salt (4%). The growth of the colonies was observed and those isolates grown on MHA plate containing antibiotic is considered as phenotypically resistant to oxacillin.

The primers used in the present study are listed in table 1. All the PCRs were performed using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma). The isolates were subjected to the PCR amplification of 23S rRNA for species confirmation with the following PCR conditions: 94°C for 5 min (initial denaturation) followed by 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 45s and then a final extension step of 72°C for 10 min (Shome et al., 2011). The purity of all the PCR products was checked on 1.5% agarose gel containing ethidium bromide (10 µg ml^{-1}). Another uniplex PCR was recruited for the genotypic detection *mecA* gene with the PCR conditions: 40 cycles of 90°C for 30s, 55°C for 30s, 72°C for 1 min and a final extension of 72°C for 5 min (Lee, 2003). DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Italy) according to the manufacturer's instructions. The same DNA was used for all the PCR reactions.

2.4 Screening of toxin genes

Uniplex PCR was employed for the molecular detection of panton-valentine leukocidin (*pvl; lukS-PV and lukF-PV*) genes with the PCR conditions: 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min (Lina et al., 1999). Three multiplex PCRs were performed for the screening of staphylococcal enterotoxins, exfoliative toxins and toxic shock syndrome toxin-1 (*sea*, *seb*, *sec, sed*; multiplex 1, *see seg*, *seh*, *sei;* multiplex 2*, tst, eta and etb;* multiplex 3) with the thermal conditions: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (Jarraud et al., 1999).

2.5 Screening of biofilm-associated genes

Biofilm forming potential of the isolates were preliminarily assessed using congo red agar (CRA) test (Bose et al., 2009). The CRA positive isolates were further subjected to PCR amplification of biofilm-associated genes. Multiplex PCR (multiplex 4) was employed for the molecular detection of *clfA, fib* and *fnbB* with PCR conditions: an initial denaturation at 94°C for 5 min followed by 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (Tristan et al., 2003). Uniplex PCRs were employed for the screening of *icaA* and *icaD* with the following thermal conditions: 95°C for 5 min (initial denaturation), 30 cycles of 95°C for 30s, 49°C for 45s, 72°C for 1 min and then a final extension of 72°C for 7 min (Notcovich et al., 2018) *icaB* (30 cycles of 94°C for 30s, 52°C for 1 min and 72°C for 1.5 min), *icaC* (30 cycles of 94°C for 30s, 50°C for 1 min and 72°C for 1.5 min) and *clfB* (initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 40s, 57°C for 50s, 72°C for 50s and a final extension at 72°C for 10 min) genes (Kiem et al., 2004; Tang et al., 2013).

2.6 Epidemiological typing

The staphylococcal protein A (*spa*) gene repeats were amplified using the standard primers and the following thermal conditions: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min, then a final extension at 72°C for 5 min (Hashemizadeh et al., 2020). Further, the *spa* type was assigned by using the available online database (http://spatyper.fortinbras.us/).

SCC*mec* typing (multiplex 5) was performed as per the standard protocol. PCR conditions were as follows; 94°C for 4 min (initial denaturation), 30 cycles of 94°C 30s, 53°C for 30s and 72°C for 1 min, and a final extension at 72°C for 4 min (Milherico et al., 2007). SCC*mec* type was predicted based on the PCR amplification of different genes in *mec* and *ccr* gene complexes.

Accessory gene regulator (*agr*) typing (multiplex 6) was performed using a common forward primer (*pan*) and four different reverse primers (*agr1*, *agr2*, *agr3* and *agr4*) to distinguish the isolates to four different *agr* groups with the following thermal conditions: 1 cycle of 94°C for 5 min followed by 26 cycles of 94°C for 30s,

55°C for 30s and 72°C for 1 min and a final extension at 72°C for 10 (Ziasistani et al., 2019).

Multi-locus sequence typing (MLST) analysis was performed by sequencing internal fragments of seven housekeeping genes; carbamate kinase (*arc*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) with the following conditions: 95°C for 5 min (initial denaturation, 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 30s, then a final extension at 72°C for 5 min (Enright et al., 2000). Sequence types (STs) were assigned by comparison with the *S. aureus* MLST database (http://www.pubmlst.org/).

3. Results

3.1 Pervasiveness of S. aureus and MRSA in fish samples

Out of 173 fish samples screened, 95 (54.9%) tested positive for *S. aureus*. A total of 95 non-duplicate bacterial isolates were identified as *S. aureus* by BD Phoenix M50 instrument which were further confirmed by the PCR amplification of 23S rRNA sequence specific to *S. aureus*. At the site level, 28 bacterial isolates of *S. aureus* (29.47%) were recovered from site 1, 34 (35.79%) from site 2 and 33 (34.74%) from site 3. Out of these 95, 23 (24.21%) isolates were found to be non-susceptible to oxacillin as evidenced by the growth on the MHA plate supplemented with oxacillin and recognized as MRSA. The molecular basis of the methicillin-resistance phenotype was determined by PCR amplification of the *mecA* (533 bp) locus. Interestingly, at the site level, the majority of MRSA (20/23, 86.96%) isolates were recovered from site 2 whereas the remaining (3/23, 13.04%) isolates were from site 1 and none from site 3.

3.2 AMR profile of MRSA isolates

The resistance profile of isolates is listed in table 2. The isolates exhibited high levels of resistance (23/23, 100%) to ampicillin, cefazoline, cefoxitin, norfloxacin, oxacillin and penicillin. In addition, resistance to non-beta-lactam

antibiotics such as erythromycin (6/23, 26.09%), gentamycin (18/23, 78.26%) and clindamycin (1/23, 4.35%) was found. The commonest resistance profile was: ampicillin (AMP)-cefazolin (CFZ)-cefoxitin (FOX)-gentamicin (GEN)-norfloxacin (NOR)-oxacillin (OXA)-penicillin (PEN) (17/23, 73.91%) with a multiple antibiotic resistance (MAR) index of 0.35. The next most common pattern was: AMP-CFZ-FOX-erythromycin (ERY)-NOR-OXA-PEN with the same MAR index, observed in 4 (17.39%) isolates. However, two isolates had a slight elevation in MAR index and this was reported to be 0.4. On a positive note, all the isolates were susceptible to linezolid and vancomycin. The percentage of resistant isolates is illustrated in Figure 1.

3.3 Carriage of genes associated with virulence and biofilm

Presence of genes associated with biofilm formation and toxicity is shown in table 2. Biofilm formation ability of MRSA was phenotypically confirmed by the CRA method, where the isolates appeared as typical black colonies (slime-producing) on a CRA plate. Further, the isolates were subjected to PCR amplification of biofilm-associated genes and it was observed that all the isolates (23/23, 100%) were harbouring *icaA* (188 bp), *icaD* (198 bp), *fib* (404 bp) and *clfB* (968 bp) genes whereas some of the isolates (18/23, 78.26%) were found to harbour the *fnbB* (524 bp) gene as well. The presence of toxin and biofilm-associated genes at the site levels are plotted in figure 2.

Molecular assessment of toxigenic potential revealed that majority of the isolates (21/23, 91.30%) carried the *pvl* gene (*lukS*-PV and *lukF*-PV, 433 bp). In addition, all the isolates (23/23, 100%) were found to harbour staphylococcal enterotoxins such as *seb* (478 bp), *seg* (642 bp) and *sei* (576 bp) genes.

3.4 Molecular typing: MLST, SCCmec, spa, and agr.

MLST analysis revealed that all the isolates (23/23, 100%) belonged to ST88 with allelic profile, 22-1-14-23-12-4-31 (Isolate details are available at PubMLST with isolate IDs 35814-35836). SCC*mec* typing was able to group a few isolates (4/23, 17.39%) to type IV while the remaining (19/23, 82.61%) isolates were non-typeable using this method. Notably, all the isolates except two belonged to *spa* type t2526

(*spa* repeat succession: 07-12-21-17-13-13-13-34-33-13) (21/23, 91.30%); a *spa*-type could not be assigned to these remaining two isolates. The *spa* locus was sequenced and aligned using BioEdit 7.2.5 version. The nucleotide sequences were supplied as supplementary file 1. The *agr* typing recognized type I (5/23, 21.74%) and type III (18/23, 78.26%) as the predominant alleles among the isolates.

4. Discussions

This study reports the incidence of MRSA in fish samples collected from three distinct study sites in a single city in Assam, India. Molecular assessment of virulence, biofilm-forming potential and epidemiological characteristics was also determined.

Northeast India has been recognized as one of the most diverse icthyofaunal hotspots and is marked by the notable biodiversity of freshwater fishes (Acharjee et al., 2012). The majority of the population (90-95%) of Assam consider fish as the primary source of protein due to its nutritive value and ready availability (Sivaraman et al., 2020). In the present study, the fish samples were collected largely from markets and beels. Fish such as Rohu (*Labeo rohitha*), silver carp (*Hypopthamychthys molitrix*), red-bellied piranha (*Pygocentrus nattereri*) and *Pungasius* sp, collected from markets came either from aquaculture or had been imported from states including Andhra Pradesh, West Bengal and Orissa whereas singari (*Mystus tengara*), aree (*Sperata seenghala*) and kawoi (*Anabas testudineus*), collected from beels, are indigenous varieties. In Assam, like anywhere else, the environmental water resources are prone to deterioration under anthropogenic influences. Additionally, highly hazardous hospital wastewater carrying many pollutants such as antibiotics, radioactive isotopes, heavy metals, cotton particles and disinfectants are discharged into natural water bodies leading to health associated complications (Kaur et al., 2020). In the present study, of the 23 fish samples tested positive of MRSA, the majority (17/23, 73.91%) of these represent river caught varieties while a few (6/23, 26.09%) represent either aquaculture (n=4) or imported (n=2) varieties. In India, prevalence rate of MRSA in fish and fish products has been reported to be 6%-11% (Vaiyapuri et al., 2019). The prevalence of MRSA in fish products is driven by several such factors as post-harvest

contamination and poor hygienic practices followed by the handlers etc (Sergelidis et al., 2014; Murugadas et al., 2017). However, its incidence in unprocessed fishes may be attributable to contamination from the surrounding environment. It is suspected that natural water bodies such as rivers, beels etc. are the hub for the hospital and industrial effluents. Pharmaceutical pollutants, on reach such water bodies, may trigger the pathogenic bacteria to develop resistance. In this context, proximity of hospitals to the water bodies in Assam certainly raises questions regarding its potential for being the source of infectious and/or drug resistant pathogens. At the same time, the incidence of MRSA in imported and cultured fish varieties hints at the possibility post-harvest contamination.

This study confirmed the presence of the *mecA* gene in all (23/23, 100%) MRSA isolates. In addition to oxacillin, resistance to ampicillin (100%), cefazolin (100%), cefoxitin (100%), penicillin (100%), norfloxacin (100%), erythromycin (26.09%), gentamycin (78.26%) and clindamycin (4.35%) was also observed. In the present study, MAR indices of the studied MRSA isolate was found to fall at 0.35 and 0.4. Generally, the MAR index may help in identifying the source of an organism in such a way that an MRSA having a MAR index of greater than 0.2 is suspected to originate from environmental samples that have had recent exposure to antibiotics (Yakubu et al., 2020). This might suggest that our MRSA may have an environmental origin. It seems likely that anthropogenic influences such as hospital effluents, contaminating the water bodies sampled cannot be discounted. Interestingly, each isolate exhibited resistance to more than three classes of antibiotics. Using a commonly used definition, a bacterial strain is said to be multidrug-resistant if it displays resistance to ≥3 classes of antibiotics (Magiorakos et al., 2012). In this context, it was disturbing to report the incidence of multidrug-resistant (MDR) *S. aureus*. On the bright side, none of the isolates exhibited resistance to teicoplanin, vancomycin and linezolid.

Bacteria, in general, do not favour existing in a planktonic state and as a result, the cells tend to attach over solid surfaces and accumulate in multi-layered cell clusters called biofilms (Azara et al., 2017). *S. aureus* is no exception and its potential to synthesis biofilm has been identified as a part of normal life cycle. *S.*

aureus in biofilm has an advantage as it can resist bactericidal activity of many conventional drugs as well as environmental stress, which is nearly impossible for planktonic bacterial cells (Chen et al., 2020). Apparently, biofilm formation has a significant role in defining pathogenicity, chronicity and irreducibility of the infection (Arciola et al., 2015). The poly intercellular adhesin (PIA) is a key protein encoded by the *icaADBC* locus that determines biofilm formation potential of *S. aureus*. In addition, other several genes such as *clfA*, *clfB*, *fnbpA*, *fnbpB*, *fib*, *eno*, *sdrC*, *sdrD*, *sdrE*, *bap* etc. are also involved. In the present study, we documented the presence of *icaAD* locus, *fib, fnbB* and *clfB*. Our findings were in agreement with several accumulating reports that have identified biofilm-associated genes in *S. aureus* (Atshan et al., 2012; Ghasemian et al., 2016; Azmi et al., 2019). All the isolates in the present study, irrespective of their epidemiological type and source of isolation, harboured *icaAD* locus, *clfB* and *fib.* However, only 18 out of 23 isolates were found to harbour *fnbB* gene. Interestingly, those isolates, which are devoid of *fnbB* locus, fell under *agr* I type.

Generally, several surface components and extracellular proteins define the toxigenic potential of *S. aureus*. The present study unveiled the toxigenic potential of MRSA isolated from fish samples by detecting the presence of *pvl*, *seb*, *seg* and *sei*. Here, presence of the *pvl* gene poses serious challenges as it is involved in pore formation in the membranes of host defence cells. It is driven by the synergistic action of two such secretory proteins as *LukS-PV* and *LukF-PV* (Melles et al., 2006). Incidence of *pvl* gene is a characteristic feature of CA-MRSA but the presence of the *pvl* gene alone cannot help in the categorization of the isolates as CA-MRSA. Here, we reported the occurrence of *pvl* gene in 21 isolates of 23 tested. A very recently published article substantiated this finding by recovering *pvl* positive MRSA from edible marine fish and recognized this as CA-MRSA (Fri et al., 2020). In addition, *S. aureus* has received substantial contributions from staphylococcal enterotoxins to establish the toxigenic potential. *S. aureus* TSS-1 (TSST-1) was perhaps the first toxin to be reported that is involved in toxic shock syndrome (TSS), characterized by episodes of multiple organs failure, fever, arterial hypertension and scarlatiniform rash, in menstrual as well as non-menstrual cases. Nevertheless, the current study reported the nonappearance of TSST-1 but did report *seb*, which is thought to be

involved in non-menstrual TSS. In addition to *seb*, incidence of *seg* and *sei* were also documented. A study reported that infection by *S. aureus* lacking TSST-1, *sea-see*, *seh*, *eta* and *etb* genes, could also result in human TSS and SSF and was attributable to the coexistence of *seg* and *sei* genes (Jarraud et al., 1999). The occurrence of seb is also reported elsewhere in *S. aureus* associated with bovine mastitis (Grispoldi et al, 2019).

In the present study, molecular epidemiology of the isolates was assessed to understand the evolutionary relationship. MLST analysis revealed that all MRSA strains belonged to ST88. MRSA belonging to ST88 has been predominantly reported from African countries and thus the establishment of such clones as "African CA-MRSA" may eventually happen in the near future (Kpeli et al., 2017). The unique survival advantage of this particular clone in this environment was evidenced by its high prevalence rate which may be driven by the unique selection pressure. The present study reported the incidence of four (17.39%) isolates belonging to ST88-IV type. Of four isolates, two (8.7%) were harbouring the *pvl* gene, which is a key feature of CA-MRSA, while the remaining two isolates were designated as *pvl*-negative SCC*mec* IV clones. Additionally, four ST88-IV isolates (both *pvl*-positive and *pvl*-negative) were resistant to erythromycin (macrolide) and norfloxacin (fluroquinolone) in addition to beta-lactam antibiotics. Nevertheless, the finding aligns, except for a resistance observed towards norfloxacin, with the reports published previously that accentuated the CA-MRSA possession of type IV SCC*mec* and its susceptibility to most of the antibiotics except macrolides and beta-lactam antibiotics (Chambers and DeLeo, 2009). To the best of our knowledge and according to the data available at the PubMLST (pubmlst.org) portal, the present study is the first report on the incidence of the ST88-IV CA-MRSA clone from India. Regarding the 19 remaining isolates, even though they carried the *pvl* locus they could not be typed by SCC*mec* and this confounded the possibility of them being CA-MRSA. The *spa* type t2526 documented in the present study has already been known to be involved in human infections (Mistry et al., 2016). According to the Ridom SpaServer, the prevalence rate of t2526 is reported to be significantly less (0.01%) and only a few reports are available, particularly from India. Mistry et al

(2016) have reported *spa* type t2526 from clinical isolates of oxacillin-sensitive, *mecA* positive *S. aureus* recovered from mastitis-affected cow's milk. Interestingly, the ST88-t2526 clone of MRSA has been reported in bovine milk samples from different countries but not from India (Hata et al., 2010; Boss et al., 2016). Thus, the present study also represents the first report on the incidence of ST88 MRSA in aquatic environs.

5. Conclusion

The present study was proposed to investigate the presence of MRSA in aquatic environs in Assam, India. A total of 173 fish samples were subjected to microbial analysis and 23 samples were recorded to harbour MRSA. Owing to the resistance of the isolates to more than 3 classes of antibiotics, the resistance profile regarded all the MRSA isolates and MDR. Toxic genes such as *pvl*, *seb*, *seg* and *sei* were detected in most of the isolates. Biofilm forming potential of the isolates were uncovered by PCR amplification of *icaAD* locus, *clfB*, *fib* and *fnbB*. Four isolates were identified as ST88-IV, which is assumed to be more prevalent in African countries. Only one spa type; t2526 was predominant irrespective of the source of isolates. In spite of such shortcomings as a lower number of MRSA isolates considered, the present study is likely to be the first report on the incidence of ST88-IV clone from India, particularly in aquatic environment. The study also underpinned the possibility of hospital waste water as the primary source of contamination and its role in the dissemination of CA-MRSA. However intensive surveillance studies will have to be performed, covering a sufficient number of samples that could represent the actual population of MRSA in the sector, to get more insight on the incidence of CA-MRSA in aquatic environments.

Acknowledgement

Authors acknowledge Department of Biotechnology, Govt. of India and Economic and Social Research Council, UK for the financial assistance. First, second and third authors acknowledge Director, ICAR- CIFT, Cochin for providing lab facilities and Dr. Rajendran Thomas, Senior Scientist, ICAR- National Research Centre on Pig, Assam for sharing the lab facility to process the samples for microbial

isolation. Scientists and Staffs of MFB Division are also deeply acknowledged by first, second and third authors for their contributions to the study.

Funding

This work was supported by Department of Biotechnology (DBT), Government of India (BT/IN/indo-UK/AMR/06/BRS/2018-19) and Economic and Social Research Council, UK.

Declarations of interest

None

Declaration of submission

All the authors approved the submission of this manuscript. All the authors declare that this manuscript has not been submitted anywhere and is not under consideration by any journals. If the manuscript is published, it will not be submitted anywhere in the same form, in English or in any other language.

Author contributions

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Table 1. List of primers used in the study

Sl No	Gene	Primer sequence	Product size (in bp)	PCR type	Annealing temperature
I	***Staphylococcus aureus* identification**				
1)	23S rRNA	AGC GAG TCT GAA TAG GGC GTT T	894	Uniplex	60°C
		CCC ATC ACA GCT CAG CCT TAA C			
II	**Amplification of *mecA* locus**				
2)	*mecA*	AAA ATC GAT GGT AAA GGT TGG C	533	Uniplex	55°C
		AGT TCT GCA GTA CCG GAT TTG C			
III	**Toxin genes**				
3)	*pvl* (*lukS*-PV; *lukF*-PV)	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	433	Uniplex	55°C
		GCA TCA AST GTA TTG GAT AGC AAA AGC			
4)	*sea*	TTGGAAACGGTTAAAACGAA	120	multiplex 1	55°C
		GAA CCT TCC CAT CAA AAA CA			
5)	*seb*	TCG CAT CAA ACT GAC AAA CG	478		
		GCA GGT ACT CTA TAA GTG CC			
6)	*sec*	GCA TAA AAG CTA GGA ATT T	257		
		AAA TCG GAT TAA CAT TAT CC			
7)	*sed*	CTA GTT TGG TAA TAT CTC CT	317		
		TAA TGC TAT ATC TTA TAG GG			
8)	*see*	CAA AGA AAT GCT TTA AGC AAT CTT AGG CCA C	482	multiplex 2	55°C
		CTT ACC GCC AAA GCT G			
9)	*seg*	AAT TAT GTG AAT GCT CAA CCC GAT C	642		
		AAA CTT ATA TGG AAC AAA AGG TAC TAG TTC			
10)	*seh*	CAA TCA CAT CAT ATG CGA AAG CAG	375		
		CAT CTA CCC AAA CAT TAG CAC C			
11)	*sei*	CTC AAG GTG ATA TTG GTG TAG G	576		
		AAA AAA CTT ACA GGC AGT CCA TCT C			
12)	*tst*	ATG GCA GCA TCA GCT TGA TA	350	multiplex 3	55°C
		TTT CCA ATA ACC ACC CGT TT			
13)	*eta*	CTA GTG CAT TTG TTA TTC AA	119		
		TGC ATT GAC ACC ATA GTA CT			
14)	*etb*	ACG GCT ATA TAC ATT CAA TT	200		
		TCC ATC GAT AAT ATA CCT AA			
IV	**Biofilm-associated genes**				
15)	*icaA*	ACACTTGCTGGCGCAGTCAA	188	Uniplex	49°C
		TCTGGAACCAACATCCAACA			
16)	*icaD*	ATG GTC AAG CCC AGA CAG AG	198	Uniplex	49°C

		AGT ATT TTC AAT GTT TAA AGC AA			
17)	*icaB*	AGA ATC GTG AAG TAT AGA AAA TT	880	Uniplex	52°C
		TCT AAT CTT TTT CAT GGA ATC CGT			
18)	*icaC*	ATG GGA CGG ATT CCA TGA AAA AGA	1066	Uniplex	50°C
		TAA TAA GCA TTA ATG TTC AAT T			
19)	*clfA*	ATT GGC GTG GCT TCA GTG CT	292	multiplex 4	55°C
		CGT TTC TTC CGT AGT TGC ATT TG			
20)	*fib*	CTA CAA CTA CAA TTG CCG TCA ACA G	405		
		GCT CTT GTA AGA CCA TTT TCT TCA C			
21)	*fnbB*	GTA ACA GCT AAT GGT CGA ATT GAT ACT	524		
		CAA GTT CGA TAG GAG TAC TAT GTT C			
22)	*clfB*	CAC TTA CTT TAC CGC TAC TTT C	968	Uniplex	57°C
		AAC GAG CAA TAC CAC TAC AAC AG			
V	**SCC*mec* typing**				
23)	CIF F2	TTC GAG TTG CTG ATG AAG AAG G	495	multiplex 5	53°C
	CIF R2	ATT TAC CAC AAG GAC TAC CAG C			
24)	ccrC F2	GTA CTC GTT ACA ATG TTT GG	449		
	ccrC R2	ATA ATG GCT TCA TGC TTC AC			
25)	RIF5 F10	TTC TTA AGT ACA CGC TGA ATC G	414		
	RIF5 F13	ATG GAG ATG AAT TAC AAG GG			
26)	SCCmec VJ1F	TTC TCC ATT CTT GTT CAT CC	377		
	SCCmec VJ1R	AGA GAC TAC TGA CTT AAG TGG			
27)	dcs F2	CATCCATATGATAGCTTGGTC	342		
	dcs R1	CTA AAT CAT AGC CAT GAC CG			
28)	ccrB2 F2	AGT TTC TCA GAA TTC GAA CG	311		
	ccrB2 R2	CCG ATA TAG AAW GGG TTA GC			
29)	kdp F1	AAT CAT GTG CCA TTG GTG ATG	284		
	kdp R1	CGA ATG AAG TGA AAG AAA GTG G			
30)	SCCmec III J1F	CAT TTG TGA AAC ACA GTA CG	243		
	SCCmec III J1R	GTT ATT GAG ACT CCT AAA GC			
31)	mec I P2	ATC AAG ACT TGC ATT CAG GC	209		
	mec I P3	GCG GTT TCA ATT CAC TTG TC			
32)	mecA P4	TCC AGA TTA CAA CTT CCC AGG	162		
	mecA P7	CCA CTT CAT ATC TTG TAA CG			
VI	**Staphylococcal protein A (*spa*) typing**				
33)	*spa*-113f	TAA AGA CGA TCC TTC GGT GAG C	Variable	Uniplex	55°C
	spa-1514r	CAG CAG TAG TGC CGT TTG CTT			
VII	**Accessory gene regulator (*agr*) typing**				
34)	*Pan*	ATG CAC ATG GTG CAC ATG C	---		
35)	*agr1*	GTC ACA AGT ACT ATA AGC TGC GAT	441		

36)	*agr2*	TAT TAC TAA TTG AAA AGT GGC CAT AGC	575	multiplex 6	55°C
37)	*agr3*	GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	323		
38)	*agr4*	CGA TAA TGC CGT AAT ACC CG	659		
VIII	**Multilocus sequence typing (MLST)**				
39)	*arcC*	TTG ATT CAC CAG CGC GTA TTG TC	Variable	Uniplex	55°C
		AGG TAT CTG CTT CAA TCA GCG			
40)	*aroE*	ATC GGA AAT CCT ATT TCA CAT TC		Uniplex	
		GGT GTT GTA TTA ATA ACG ATA TC			
41)	*glp*	CTA GGA ACT GCA ATC TTA ATC C		Uniplex	
		TGG TAA AAT CGC ATG TCC AAT TC			
42)	*gmk*	ATC GTT TTA TCG GGA CCA TC		Uniplex	
		TCA TTA ACT ACA ACG TAA TCG TA			
43)	*pta*	GTT AAA ATC GTA TTA CCT GAA GG		Uniplex	
		GAC CCT TTT GTT GAA AAG CTT AA			
44)	*tpi*	TCG TTC ATT CTG AAC GTC GTG AA		Uniplex	
		TTT GCA CCT TCT AAC AAT TGT AC			
45)	*yqiL*	CAG CAT ACA GGA CAC CTA TTG GC		Uniplex	
		CGT TGA GGA ATC GAT ACT GGA AC			

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