Title: Methicillin-resistant *Staphylococcus haemolyticus* (MRSH) in fish samples harboring atypical staphylococcal cassette chromosome *mec* (SCC*mec*) elements.

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31 **Abstract**

32 Incidence of *Staphylococcus haemolyticus* in fish is a sign of post-harvesting
33 contamination. The main objective of the present study was to monitor the prevalence of
34 methicillin-resistant *Staphylococcus haemolyticus* (MRSH) in fishes and its genotypic
35 characterization. Fish samples (n=79) were collected from retail markets in three pre-decided
36 sites in Assam. The collected fish samples were subjected to microbial analysis and as a result
37 13 MRSH were isolated. Susceptibility of the isolates towards 13 classes of antibiotics was
38 assessed by employing BD Phoenix M50 system. Polymerase chain reaction (PCR) was
39 performed for the molecular detection of methicillin-resistance determinant. Molecular typing
40 of the isolates was attempted by recruiting staphylococcal cassette chromosome *mec*
41 (SCC*mec*) and pulsed-field gel electrophoresis (PFGE). The studied isolates showed varying
42 levels of resistance to different classes of antibiotics such as cephalosporin (100%),
43 lincosamide (30.76%), macrolides (30.76%), aminoglycoside (53.85%), quinolones (38.46%)
44 and sulfonamides (61.54%). BD Phoenix M50 instrument recognized a few (30.76%) isolates
45 as inducible macrolide-lincosamide-streptogramin B (iMLSb) phenotype. Remarkably, all the
46 isolates were reported as multi-drug resistant (MDR) as they showed resistance to ≥3 classes
47 of antibiotics. All the studied isolates tested positive for *mecA* gene and were carrying multiple
48 SCC*mec* elements. PFGE cluster analysis grouped the isolates into two major clusters and
49 seven individual lineages. In short, the current study documented the incidence of multi-drug
50 resistant, multiple SCC*mec* elements carrying *S. haemolyticus* in fish samples. The present
51 study underpinned the significance of enhanced surveillance of MRSH and also the role of the
52 hygiene to mitigate the AMR in fisheries.

53 **Key words**: multi-drug resistance, PFGE, SCC*mec* elements, methicillin-resistant
54 *Staphylococcus haemolyticus*, Northeast India.

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66 **Introduction**

67 *Staphylococcus haemolyticus* is a coagulase-negative bacterium found commonly in
68 anterior nares, ear canals, inguinal areas, respiratory and gastrointestinal mucosal membranes
69 of humans as well as animals (Piette and Verschraegen 2009). Among coagulase-negative
70 staphylococci (CoNS), *S. haemolyticus* is only second to *S. epidermidis* in causing bloodstream
71 infections (Barros et al., 2012). Generally, CoNS is considered as less virulent or sometimes
72 non-virulent since it lacks coagulase, an enzyme-like protein which determines the invasive
73 pathogenic potential of staphylococci (Prasad et al., 2012). Nevertheless, many studies
74 documented the emergence of *S. haemolyticus* as an opportunistic pathogen which can cause
75 severe infections in immunocompromised, immunosuppressed patients and those with
76 underlying diseases (Soumya et al., 2017). Additionally, the potential of *S. haemolyticus* to
77 develop resistance against conventional drugs is also a growing concern. Antimicrobial
78 resistance (AMR), the ability of bacteria to encompass resistance against drugs, is one of the
79 hot topics of recent times and has gained momentum as annual mortality rates have
80 surprisingly elevated to unprecedented levels (Hendriksen et al., 2019). It is estimated that the
81 mortality attributed to AMR may hit 10 Million by 2050 if the scenario is poorly addressed
82 (Cassini et al., 2019). Methicillin-resistance in staphylococci is greatly conferred by the
83 acquisition of *mecA* gene which is carried on a mobile genetic element, namely Staphylococcal
84 Cassette Chromosome *mec* (SCC*mec*) (Sekizuka et al., 2020). However, in addition to the
85 *mecA*, a homologue namely $mecA_{LGA251}$ has also been reported (Stegger et al., 2012). *S.*
86 *haemolyticus* is the best example of a pathogen that has evolved spectacularly to acquire
87 multi-drug resistance owing to the extreme plasticity of its genome (Chiew et al., 2007).
88 SCC*mec* elements, owing to their high transmissibility, contribute greatly to the dissemination
89 of methicillin resistance among susceptible staphylococci by horizontal gene transfer
90 (Chongtrakool et al., 2006). Delineation of closely related bacterial isolates is essential for
91 epidemiological surveillance. Pulsed-field gel electrophoresis (PFGE), a gold standard used
92 predominantly in molecular typing is a reliable method for the delineation of staphylococci.
93 PFGE is essentially employed when there is a disease outbreak and it enables source tracking
94 among outbreak isolates (Bannerman et al., 1995). PFGE also helps to explicate the genetic
95 diversities among isolates (Ruiz et al., 2008).

96 In Assam, fishery plays very crucial role as it provides livelihood for billions of people
97 and to pursue their daily requirements. Considering the fact that more than 2% of gross state
98 domestic product (GSDP) to the state economy is contributed by the fishery, significance of
99 the sector particularly in Assam is well understood. In fact, the peculiar sub-tropical climate
100 and vast piscine diversity makes the state congenial for establishing the freshwater fish
101 cultures (Gogoi et al., 2015). It was noted with surprise that the 95% of total population in
102 Assam are fish eaters and that underlined the high demand of fishes in markets. Hence, it has
103 been forced to increase the fishery production and as a result the state has secured sixth
104 position in the country as inland fish producers. As far as the aquatic resources of Assam as a

source of food is concerned, the piscine diversity is commendable since out of 216 species of fishes reported till now, 210 have nutritive value (Gogoi et al., 2015). On the other hand, scarcity of people's involvement, unsustainable utilization of aquatic resources and inappropriate farming system approaches (FSA) pose challenges to the growing fishery sector in Assam. Unscientific farming methods can provide ideal environments for bacterial contamination.

MRS in context of nosocomial as well as community-associated infections has been discussed widely in the literature. Studies are outnumbered that indicated the prevalence of staphylococcus in hospitals and health-care sectors (Becker et al., 2014). Similarly, many articles documented the prevalence of MRSH in companion animals and its dissemination to humans (Ruzauskas et al., 2014). Reports are not less, when comes to its prevalence in environmental samples (Dziri et al., 2016). However, all these studies are reflecting the incidence of CoNS in different sectors and potentiality of being reservoir for the intra- and inter-sectoral dissemination of the bacteria. On the contrary, only a few studies have demonstrated the incidence of methicillin-resistant staphylococci other than *S. aureus,* particularly in environmental samples (Pokhrel et al., 2018). Thus, considering these facts, it appeared rational to monitor the prevalence of MRS in fish samples from natural water bodies and local markets. In this study, we investigated the incidence of MRSH and its molecular characterization in the selected sites in Assam, India.

Materials and methods

Sampling strategy

Sampling was performed in August, 2019 at three distinct locations in Assam (Northeast India) *viz*., 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). All the three sites were at a distance of less than 26 km radius from the center of the city (Fig 1).

A total of 79 samples comprising diverse species of fish fauna, are collected from retail markets and natural water bodies in these sites. Of 79 fish samples, 17 were collected from site 1, 21 from site 2 and remaining 41 from site 3. Samples were preserved in a sterile polythene bag on ice and transported to the laboratory in chilled condition.

Sample processing and bacterial isolation

Samples were initially processed at Quality Control lab, ICAR- National Research Centre on Pig (ICAR-NRCP), Rani, Assam, India. Microbial identification and further analyses were performed at Microbiology Fermentation and Biotechnology Division of ICAR-Central Institute of Fisheries Technology (ICAR-CIFT), Cochin, Kerala, India.

Standard protocol of United States Food and Drugs Administration (USFDA) were followed to process fish samples for the isolation of Staphylococci with slight modifications

142 (Bennet and Lancet 2001). Briefly, 25 grams of each sample were aseptically transferred to
143 225 mL Trypticase soy broth (TSB) (BD BBL and Difco, USA) supplemented with 10% (w/v)
144 sodium chloride and 1% (w/v) sodium pyruvate and incubated at 37°C for overnight. Enriched
145 cultures were then serially diluted and 200 µL inoculated on mannitol salt agar (MSA) (BD BBL
146 and Difco, USA) by spread plate method followed by incubation at 37°C for 48 hours. Mannitol
147 non-fermenting colonies (pink color) with colony characteristics of CoNS were picked to screen
148 the methicillin resistance as stated by The Clinical and Laboratory Standards Institute (CLSI)
149 guidelines (CLSI 2020). Presumptive CoNS (n=5) colonies from each MSA plate were
150 inoculated separately to Brain Heart Infusion broth (BHI) (BD BBL and Difco, USA) to prepare
151 the samples for spot test. Each sample (10 µL) were spot inoculated on Muller-Hinton agar
152 (MHA) (BD BBL and Difco, USA) supplemented with 4% (w/v) sodium chloride and Oxacillin
153 (6µg/mL) (Sigma-Aldrich, USA) followed by overnight incubation at 35°C. Those isolates with
154 reduced susceptibility to oxacillin and grown on MHA+ Oxacillin plate were discerned to carry
155 methicillin resistance determinant and considered for further studies.

156 **Biochemical characterization and Antimicrobial Susceptibility Test (AST)**

157 BD Phoenix™ M50 system (BD Diagnostic Systems, Sparks, MD) was employed for the
158 bacterial identification and to study AST profile of the selected isolates according to the
159 protocol described elsewhere (Hong et al., 2019). Briefly, Bacterial colonies were inoculated
160 into the bacterial identification broth (ID broth) at a concentration of 0.5 McFarland. Twenty-
161 five microliters of the adjusted ID broth were transferred into the AST broth (BD Diagnostic
162 Systems) with the AST indicator (methylene blue and resazurin). The prepared suspension was
163 poured through the fill port on both sides of the BD Phoenix™ PMIC/ID combo panel (BD
164 Diagnostic Systems) and is sealed, then loaded to the instrument. Test results were analyzed
165 using EpiCentre™ software. BDXpert infers the resistance level based on minimum inhibitory
166 concentration (MIC) mentioned in CLSI guidelines. Quality controls were performed according
167 to the manufacturer's recommendations using reference isolate, *S. aureus* ATCC 25923.

168 **Molecular detection of methicillin resistant determinant: *mecA* gene**

169 All amplifications were performed on supernatants from DNA lysate. To prepare the
170 lysate, 200 µL of bacterial suspension was initially heated at 94°C in AccuBlock™ digital dry
171 bath (Labnet international, USA) for exactly 10 minutes followed by immediate cooling at -
172 80°C for at least one hour. Samples after incubation were centrifuged for 5 minutes at 8500
173 rpm and the supernatant was used as DNA template. PCR was performed in a 50 µL reaction
174 volume with Red Taq®Ready Mix™PCR reaction mix (Sigma-Aldrich, USA), 800 nM
175 concentrations of *mecP4* and *mecP7* primers (Table 1) and 5 µL of DNA lysate. PCR
176 amplifications were performed in Veriti™ 96-well Thermal cycler (Applied Biosystems) with the
177 PCR conditions mentioned in table 1. PCR products (10 µL) were resolved on a 2% agarose
178 (Sigma-Aldrich, USA) gel in 1X Tris-Acetate-EDTA (TAE) buffer (Bio-Rad, Hercules, Calif) to
179 which added EtBr (0.5 µg/mL) to visualize the amplified product.

180 **Molecular typing of MRSH**

181 **SCC*mec* typing**

182 Multiplex PCR was employed for SCCmec typing (Rajan et al., 2015). Amplification was
183 performed using Red Taq® Ready Mix™ PCR reaction mix (Sigma-Aldrich, USA). Primers used
184 in this study are listed in Table 1. Amplifications were performed with the PCR condition
185 mentioned in table 1.

186 **Pulsed-Field Gel Electrophoresis (PFGE)**

187 All the 13 isolates used in the current study were genotyped by *SmaI*-pulsed field gel
188 electrophoresis. Briefly, genomic DNA was prepared in agarose plugs as described previously
189 (Rajan et al., 2015) and were subjected to restriction digestion by 40 U of *SmaI* (New England
190 Biolab, UK) for 18 h at 25°C. To ensure successful digestion, additional 20 U of *SmaI* (NEB,
191 UK) was added and incubated for 4 h at 25°C whereas marker strain was restriction digested
192 with *XbaI* (NEB, UK). PFGE was carried out in contour-clamped homogenous electric field
193 (CHEF) system (Bio-Rad, USA) with following parameter; Temperature at 14°C, initial switch
194 time for 5 seconds, final switch time for 40 seconds and for a duration of 21 hours. The gel
195 was stained with ethidium bromide (1 µg/ml) for 20 min and documented with UV imaging
196 system. *Salmonella* serotype strain H9812 of known banding pattern was used as the marker
197 strain. The total DNA was digested with *SmaI* restriction enzyme (New England Biolabs,
198 Baverly, Mass.) into several fragments separated on agarose gel. Cluster analysis was
199 performed using BioNumerics 7.6.1 software with a similarity co-efficient optimization of 0.5%
200 and band matching tolerance of 1%. The dendrogram was constructed based on un-weighted
201 pair group method with arithmetic mean (UPGMA) algorithm. Genetic diversity among MRSH
202 isolates were assessed based on dice similarity index.

203 **Statistical analysis**

204 Data were statistically analyzed using one-way Analysis of Variance (ANOVA) followed
205 by Duncan's test for testing the significance of difference.

206 **Results**

207 **Demography of fish population in Assam**

208 It is evident from many literatures that the Assam bears a spectacular piscine diversity.
209 Basically, as far as sources were concerned; the samples were classified into three such
210 categories as river-caught, cultured fishes and imported varieties. Certain fish varieties such as
211 singara (*Mystus tengara*), singhi (*Heteropneustes fossilis*) and puthi (Puntius spp.) are
212 indigenous to Assam and represents typical examples of river-caught fish varieties.
213 Aquaculture settings are well established in villages such as Nagaon and Hajo. The fish
214 varieties such as *Pangasius*, *Labeo rohitha* and *L. catla* were imported from different states. In
215 our study, the fish samples collected from natural water bodies were largely endemic to

216 Northeast India, while the majority of those collected from retail markets were imported from
217 different states of the country.

218 **Bacterial identification and resistance pattern**

219 Thirty non-duplicate MRS were recovered from 79 tested fish samples and 13 (43.33%)
220 were identified as MRSH. At the site level, prevalence of MRSH (12/13, 92.31%) was found to
221 be very high at site 3. Only 1 fish sample collected from site 1 were found to harbor MRSH.
222 The MICs of antibiotics to each studied isolates are represented in Table 2. BD Phoenix M50
223 was employed to test the isolates against a panel of 13 classes comprising 21 antibiotics. All
224 13 (100%) isolates were resistant to ampicillin, cefazolin, methicillin and penicillin, 4 (30.76%)
225 to clindamycin, 4 (30.76%) to erythromycin, 7 (53.85%) to gentamicin, 5 (38.46%) to
226 norfloxacin and 8 (61.54%) to trimethoprim-sulfamethoxazole (Fig 2). Additionally, 5
227 (38.46%) isolates showed intermediate resistance to gentamicin and 1 (7.69%) to
228 tetracycline. Based on clindamycin and erythromycin resistance, 4 (30.76%) isolates were
229 reported as inducible macrolide-lincosamide-streptogramin B (iMLSb) phenotype. On the
230 brighter side, neither vancomycin nor linezolid resistance was observed. Surprisingly, all 13
231 (100%) *S. haemolyticus* isolates were reported as MDR (resistant to ≥3 classes) (Table 2).

232 **Determination of *mecA* gene and SCC*mec* type**

233 Isolates were subjected to PCR for the molecular detection of *mecA* gene. All the 13
234 (100%) isolates of MRSH were tested positive of *mecA* gene. Furthermore, in contrast to
235 previously reported SCC*mec* types, all the isolates used in the present study revealed an
236 unusual combination of SCC*mec* cassette. Twelve (92.3%) isolates exhibited similar SCC*mec*
237 type having the amplicons corresponding to internal control; *mecA* gene (162 bp), *kdp* gene
238 (284 bp), *RIF5* gene (414 bp), *ccrC* gene (449 bp) and *CIF2* gene (495 bp) which indicated a
239 possible combination of type I, type II, type III and type V. In contrast, the 13th isolate had
240 the combination of *mecA* gene (162 bp), *RIF5* gene (414 bp) and *ccrC* (449 bp) gene,
241 indicated a mixture of type III and type IV SCC*mec* elements.

242 **PFGE typing**

243 Four isolates recovered from *Macrognathus aral*, *Puntius sophore*, *Ompok bimaculatus*
244 and *Pangasium pangasius* showed 100% similarity and grouped into a single cluster. Similarity
245 of those recovered from *Channa punctatus* and *Hypophthalmichthys molitrix* also found to be
246 100% and grouped into another cluster. However, these two clusters showed only 50%
247 similarity. Major cluster had four isolates whereas the other cluster had only two. Though
248 remaining isolates were existed as single lineages, similarity among them found to be nearly
249 90%. The isolate recovered from site 1 showed 94.75% similarity with major cluster. The
250 PFGE band patterns of the isolates and PFGE clustering are depicted in fig 3.

251 **Discussion**

252 It is not surprising to observe the occurrence of *S. haemolyticus* in humans and animals
253 since it is a ubiquitous commensal organism of normal skin flora. Nevertheless, their
254 prevalence in processed fishes is an indication of post-harvest bacterial contamination
255 (Sergelidis et al., 2014). According to Sergelidis et al, the incidence of CoNS in ready to eat
256 fishes was high mainly because of poor hygiene of fish handlers. Another study where the
257 incidence of staphylococci in edible portion of fishes was examined before and after cleaning
258 the gut, observed that the staphylococcal load was reduced after cleaning the gut (Sahoo et
259 al., 2009). The above observations further emphasized the pivotal role of cleanliness to avoid
260 post-harvest contamination. In our study, all *S. haemolyticus* were recovered from retail
261 market fishes, possibly indicating either post-harvesting contamination or poor hygienic
262 practices followed by fish vendors. In addition to *S. haemolyticus*, other staphylococcal species
263 such as *S. sciuri*, *S. xylosus*, *S. gallinarum*, *S. warneri* etc. were also identified from market
264 fishes as well as those collected from natural water bodies which are reported already as
265 major contaminants of meat and fish products (Regecová et al., 2014).

266 Susceptibility status against oxacillin revealed that all the studied MRSH isolates
267 consistently showed resistance to oxacillin. To support the credibility, our findings were
268 compared with a study conducted in Japan where reported the occurrence of methicillin-
269 resistant coagulase-negative particularly *S. haemolyticus* in retail ready to eat raw fish
270 (Hammad et al., 2012). Historically, the first case of methicillin resistance was reported in
271 1961 from United Kingdom, soon after the introduction of methicillin into clinical practice
272 (Enright 2003). In contrast to wild type staphylococci, methicillin resistant isolates have a
273 modified form of penicillin binding protein (PBP) namely PBP2a, encoded by *mecA* gene which
274 has low affinity for beta-lactam antibiotics (Stapleton and Taylor 2002). In our study, we
275 explicated the presence of gene that confers methicillin-resistance in all the MRSH isolates,
276 confirming the drug resistance is mediated by *mecA* gene. Increasing prevalence of methicillin
277 resistance necessitated the introduction of vancomycin (Kirby 1984). However, in our study
278 neither vancomycin nor linezolid resistance was encountered. In addition to oxacillin,
279 resistance to clindamycin (30.76%), erythromycin (30.76%), gentamicin (53.86%), norfloxacin
280 (38.46%) and trimethoprim-sulfamethoxazole (61.54%) was also observed. The findings were
281 in agreement with the resistance pattern of nosocomial MRSH reported elsewhere (Szczuka et
282 al., 2016). Staphylococcal isolates, when found resistant to erythromycin, can be declared as
283 clindamycin-resistant (Magiorakos et al., 2012). Similarly, when found resistant to oxacillin or
284 cefoxitin, the isolates are considered to be resistant to all beta-lactam antibiotics except anti-
285 MRSA cephalosporin. Thus, as far as the definition of MDR with respect to MRS is concerned,
286 all isolates which are resistant to methicillin is generally treated as MDR (Magiorakos et al.,
287 2012). It is noteworthy that all MRSH strains in the present study were characterized as MDR
288 not only by the definition of Magiorakos et al, 2012 but also as the isolates showed remarkable
289 resistance against more than 3 classes of antibiotics. Innate ability of *S. haemolyticus* to
290 acquire MDR is greatly attributed to its genome plasticity. Additionally, predominance of the

insertion elements (IS elements) in *S. haemolyticus* are significantly contributing to the emerging drug resistance simply by activation or inactivation of resistance determinants (Takeuchi et al., 2005).

The present study used the primers that could group the isolates only up to six SCC*mec* types. Unusual combination of SCC*mec*, documented in our study in all MRSH isolates, was tempting to recognize the isolates as either non-typeable or the mixture of multiple *mec* elements. It is indeed reported that the SCC*mec* diversities among CoNS are remarkably high and those non-typeable isolates can act as a reservoir for SCC*mec* elements (McManus et al., 2015). Several studies, over the years substantiated our findings by reporting atypical combination of SCC*mec* elements particularly in CoNS (Otto 2013; Hannson and Sollid 2007). Besides, the new combination of SCC*mec* in MRSH may contribute to epidemiological importance of the strain as they are speculated to be the reservoir of methicillin-resistance determinants which can easily be disseminated to virulent isolates of *S. aureus*. However, whole genome sequencing (WGS) of the isolates should be recruited to achieve more insight to the structural characterization of *SCCmec* elements.

Bacterial typing in general, plays a crucial role in phenotypic and genotypic discrimination of isolates especially when an outbreak is reported (Neoh et al., 2019). Previously bacterial typing was performed by implementing phage typing, serotyping and analyzing susceptibility pattern. However, PFGE gained tremendous acceptance over these techniques owing to the reproducibility since the technique is DNA based, rapidity and robustness (Adzitey et al., 2013). In this study, we employed PFGE to investigate the genetic diversity among MRSH isolates. Isolates were grouped into 2 distinct clusters showing genetic relatedness among the isolates. It is noteworthy that all isolates, recovered from one particular site (site 3) has grouped to different clusters, probably indicating the prevalence of multiple clones at this area. Our observations were comparable to a previously reported study where seven *S. haemolyticus* isolates from a hospital were grouped to same cluster (Chamon et al., 2014). Importantly, comparing DNA fingerprint of PFGE with that of SCC*mec* recognized one particular isolate as an outlier since it showed different band pattern from that of other isolates in both PFGE and SCC*mec* typing. It was also notable to observe the isolates belonging to the same cluster showed different antibiotic resistance pattern. It may be attributable to phenotypic plasticity which refers to changes in the traits of an organism in response to its microenvironment. In our study, though isolates are basically from fish samples, each fish belongs to different niches and provides a different micro-environment for the bacteria. Such micro-environment can trigger certain phenotypic changes in the organism. A recent study reported elsewhere perceived the phenotypic plasticity of *S. epidermidis* isolated from three different niches (Garcia-Gutierrez et al., 2020).

Conclusions

328 The present study outlined the incidence of multidrug resistant *S. haemolyticus* in
329 market fishes. The antibiogram revealed the potential resistance to penicillins, cephalosporins,
330 lincosamides, aminoglycosides, marcolides, quinolones and sulfonamides. The unusual
331 combination of SCC*mec* elements in the isolates contributed to the epidemiological
332 significance. PFGE analysis documented the prevalence of multiple clones at one site. Presence
333 of MRSH in market fish samples pointed at the hand contamination among food handlers as
334 the major source. Thus, the current study emphasized the paramount importance of food
335 handlers to follow strict hygienic practices. Moreover, the enhanced surveillance of antibiotic
336 use in aquaculture settings should be ensured as the imprudent antibiotic usage may
337 contribute to the emergence of AMR. However, the study has to be expanded in terms of
338 number of samples and duration of sampling that covers all the major climates of the country
339 in order to achieve more comprehension to the prevalence of AMR in fish and its dissemination
340 to different sectors.

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354 **Conflict of Interest**

355 Authors declare no conflict of interests

356 **Ethics and Approval**

357 Not applicable

358 **Consent for publication**

359 Consent has been obtained from all the authors regarding the submission of this
360 manuscript.

361 **Availability of data and material**

362 Not applicable.

363 **Author's contribution**

364 Conceptualization and supervision: G. K Sivaraman. Fund acquisition: Bibek Shome and
365 Mark Holmes. Jennifer Cole edited and reviewed the manuscript. K. H Muneeb and Sudha S
366 were involved in sample collection and Microbial processing. K. H Muneeb performed the
367 experiments and prepared first draft of the manuscript.

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514 **<u>Figures</u>**

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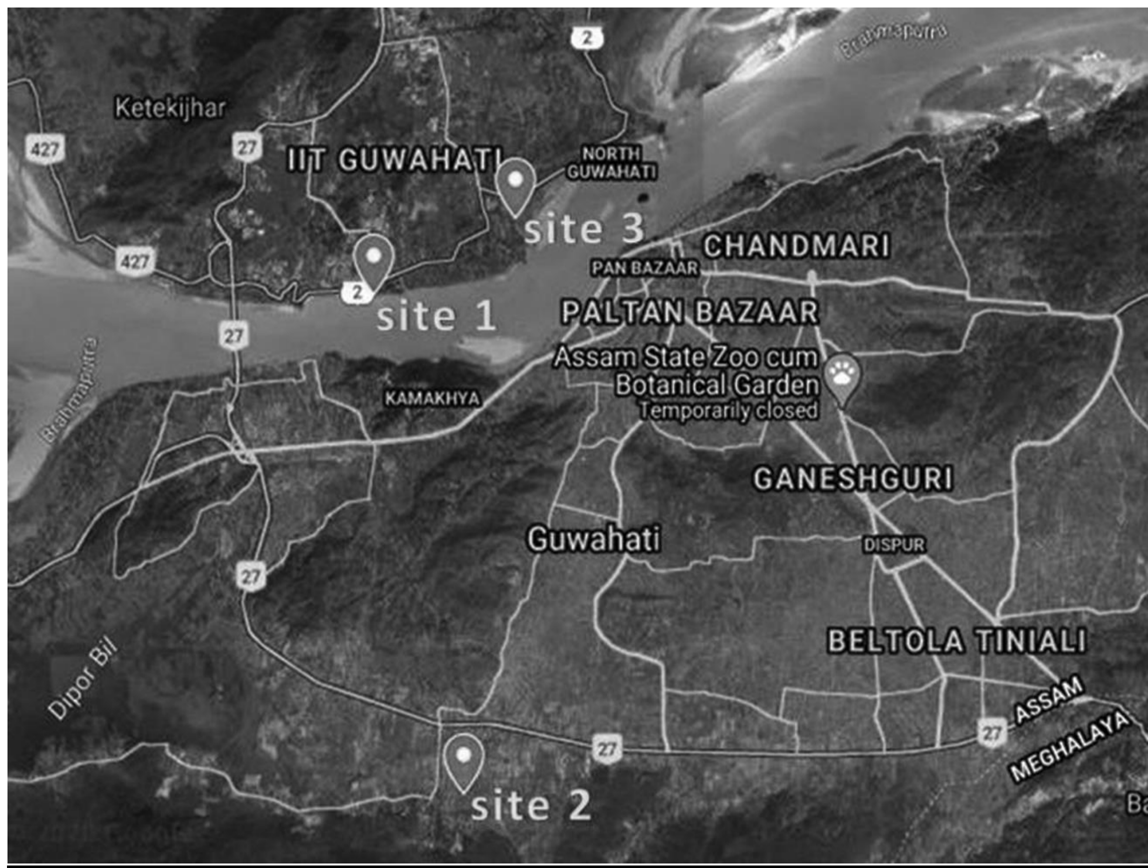

516 **Fig 1** showing such sites as site 1 (Silagrant), site 2 (Garchuk) and site 3 (North Guwahati
517 Township Committee) from where samples were drawn. Pin map symbols on the map indicate
518 the three study sites as mentioned.

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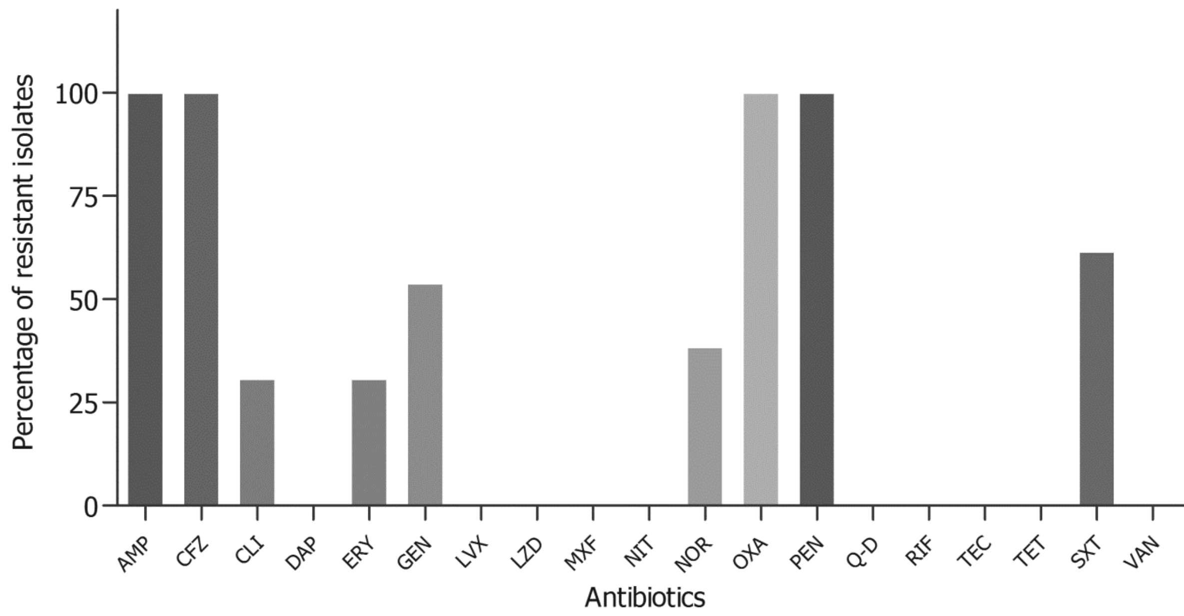

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534 **Fig 2** showing the percentage of non-susceptible isolates of methicillin resistant
535 *Staphylococcus haemolyticus* against various antibiotics.

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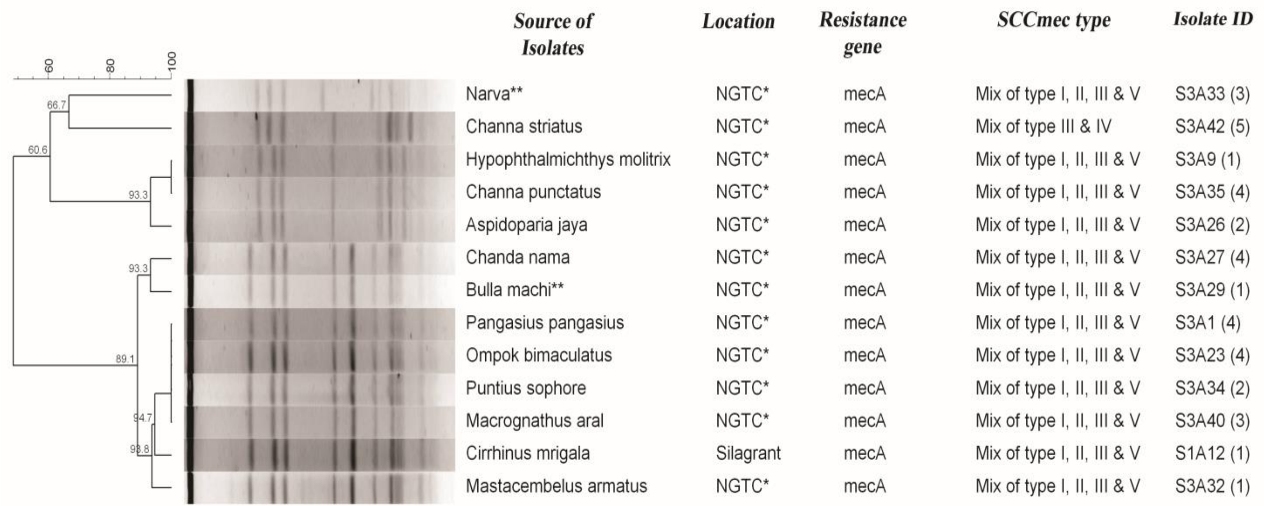

555 **Fig 3** PFGE cluster analysis of methicillin resistant *Staphylococcus haemolyticus.*

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576 **Tables**

577 **Table 1**. List of primer sequences used in the study for SCCmec typing of methicillin resistant *Staphylococcus haemolyticus* (MRSH)

Sl No	Primer name	Primer sequence	Product size (in bp)	SCCmec region	PCR conditions	Reference
1	CIF F2	TTCGAGTTGCTGATGAAGAAGG			Initial denaturation at 94°C for 15 minutes followed by 30 cycles of 30 sec at 94°C, 30 sec at 53°C and 1 min at 72°C. Final extension at 72°C for 7 min.	(Milherico et al. 2007)
	CIF R2	ATTTACCACAAGGACTACCAGC	495	I, J1 region		
2	ccrC F2	GTACTCGTTACAATGTTTGG				
	ccrC R2	ATAATGGCTTCATGCTTCAC	449	V, ccr complex		
3	RIF5 F10	TTCTTAAGTACACGCTGAATCG				
	RIF5 F13	ATGGAGATGAATTACAAGGG	414	III, J3 region		
4	SCCmec VJ1F	TTCTCCATTCTTGTTCATCC				
	SCCmec VJ1R	AGAGACTACTGACTTAAGTGG	377	V, J1 region		
5	dcs F2	CATCCATATGATAGCTTGGTC				
	dcs R1	CTAAATCATAGCCATGACCG	342	I, II, IV & VI, J3 region		
6	ccrB2 F2	AGTTTCTCAGAATTCGAACG				
	ccrB2 R2	CCGATATAGAAWGGGTTAGC	311	II & IV, ccr complex		
7	kdp F1	AATCATGTGCCATTGGTGATG	284			
	kdp R1	CGAATGAAGTGAAAGAAAGTGG		II, J1 region		
8	SCCmec III J1F	CATTTGTGAAACACAGTACG				
	SCCmec III J1R	GTTATTGAGACTCCTAAAGC	243	III, J1 region		
9	mec I P2	ATCAAGACTTGCATTCAGGC				
	mec I P3	GCGGTTTCAATTCACTTGTC	209	II & III, mec complex		
10	mecA P4	TCCAGATTACAACTTCCCAGG				
	mecA P7	CCACTTCATATCTTGTAACG	162	Internal control		

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580 **Table 2**: Minimum inhibitory concentrations (MICs) of antibiotics determined for methicillin-resistant *Staphylococcus haemolyticus* (MRSH)

Isolate ID	β-Lactams					Non β-Lactams															MDR	Source of Isolates		Resistant gene
	Cephems		Penicillin derivatives																					
	Cephalosporin I	Cephamycins	Penicillinase-labile penicillins		Penicillinase stable penicillins	Fluroquinolones			Aminoglycosides	Ansamycin	Glycopeptides		Folate pathway antagonists	Lincosamide	Lipopeptides	Macrolides	Nitroheterocyclics	Oxazolidinones	Streptogramin	Tetracyclines				
			Penicillins	Aminopenicillins						Rifamycin	Glycopeptide	Lipoglycopeptide												
	Cefazolin*	Cefoxitin*	Penicillin*	Ampicillin*	Oxacillin	Moxifloxacin	Norfloxacin	Levofloxacin	Gentamicin	Rifampin	Vancomycin	Teicoplanin	Trimethoprim-Sulfamethoxazole	Clindamycin***	Daptomycin	Erythromycin	Nitrofurantoin	Linezolid	Quinupristin-Dalfopristin	Tetracyclin		Local name	Scientific name	
S1A12 (1)	**R**	**R**	**R**	**R**	**>2 R**	**<=0.25 S**	**<=1 S**	**<=0.5 S**	**>8 R**	**<= 0.5 S**	**<= 1 S**	**2 S**	**>4/76 R**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Mrigal	*Cirrhinus mrigala*	*mecA*
S3A1 (4)	**R**	**R**	**R**	**R**	**>2 R**	**1 S**	**2 S**	**>2 X****	**8 I**	**<= 0.5 S**	**<= 1 S**	**2 S**	**<=1/19 S**	**<= 0.25 R**	**<=0.5 S**	**>4 R**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Kos	*Pangasius pangasius*	*mecA*
S3A9 (1)	**R**	**R**	**R**	**R**	**>2 R**	**<=0.25 S**	**<=1 S**	**<=0.5 S**	**>8 R**	**<= 0.5 S**	**<= 1 S**	**2 S**	**>4/76 R**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Silver Karp	*Hypophthalmichthys molitrix*	*mecA*
S3A23 (4)	**R**	**R**	**R**	**R**	**>2 R**	**<=0.25 S**	**<=1 S**	**<=0.5 S**	**>8 R**	**<= 0.5 S**	**<= 1 S**	**4 S**	**>4/76 R**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Kajali	*Ompok bimaculatus*	*mecA*
S3A26 (2)	**R**	**R**	**R**	**R**	**>2 R**	**1 S**	**>8 R**	**>2 X****	**4 S**	**<= 0.5 S**	**<= 1 S**	**4 S**	**<=1/19 S**	**<= 0.25 R**	**<=0.5 S**	**>4 R**	**<=16 S**	**<=1 S**	**<=0.5**	**8 I**	**Yes**	Borela	*Aspidoparia jaya*	*mecA*

S3A27 (4)	**R**	**R**	**R**	**R**	**>2 R**	**<=0.25 S**	**<=1 S**	**<=0.5 S**	**>8 R**	**<= 0.5 S**	**<= 1 S**	**<=1 S**	**>4/76 R**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Chanda	*Chanda nama*	*mecA*
S3A29 (1)	**R**	**R**	**R**	**R**	**>2 R**	**1 S**	**>8 R**	**>2 X****	**8 I**	**<= 0.5 S**	**<= 1 S**	**4 S**	**<=1/19 S**	**<= 0.25 R**	**<=0.5 S**	**>4 R**	**<=16 S**	**<=1 S**	**<=0.5**	**8 I**	**Yes**	Bulla machi	Not available	*mecA*
S3A32 (1)	**R**	**R**	**R**	**R**	**>2 R**	**<=0.25 S**	**<=1 S**	**<=0.5 S**	**>8 R**	**<= 0.5 S**	**<= 1 S**	**2 S**	**>4/76 R**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Bami	*Mastacembelus armatus*	*mecA*
S3A33 (3)	**R**	**R**	**R**	**R**	**>2 R**	**<=0.25 S**	**<=1 S**	**<=0.5 S**	**>8 R**	**<= 0.5 S**	**<= 1 S**	**<=1 S**	**>4/76 R**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Narva	Not available	*mecA*
S3A34 (2)	**R**	**R**	**R**	**R**	**0.5 R**	**1 S**	**>8 R**	**>2 X****	**8 I**	**<= 0.5 S**	**<= 1 S**	**<=1 S**	**<=1/19 S**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**<=0.5 S**	**Yes**	Puti	*Puntius sophore*	*mecA*
S3A35 (4)	**R**	**R**	**R**	**R**	**>2 R**	**1 S**	**>8 R**	**>2 X****	**8 I**	**<= 0.5 S**	**<= 1 S**	**4 S**	**<=1/19 S**	**<= 0.25 R**	**<=0.5 S**	**>4 R**	**<=16 S**	**<=1 S**	**<=0.5**	**8 I**	**Yes**	Khorai	*Channa punctatus*	*mecA*
S3A40 (3)	**R**	**R**	**R**	**R**	**0.5 R**	**1 S**	**>8 R**	**>2 X****	**<=2 S**	**<= 0.5 S**	**<= 1 S**	**<=1 S**	**<=1/19 S**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**<=0.5 S**	**Yes**	Tura	*Macrognathus aral*	*mecA*
S3A42 (5)	**R**	**R**	**R**	**R**	**>2 R**	**<=0.25 S**	**<=1 S**	**<=0.5 S**	**8 I**	**<= 0.5 S**	**<= 1 S**	**<=1 S**	**>4/76 R**	**<= 0.25 S**	**<=0.5 S**	**<= 0.25 S**	**<=16 S**	**<=1 S**	**<=0.5**	**2 S**	**Yes**	Sol machi	*Channa striatus*	*mecA*

*Isolates resistant to oxacillin are inevitably considered as resistant to all classes of beta-lactam antibiotics except anti-MRSA beta lactam antibiotics. Thus MIC value for beta-lactam antibiotics are not mentioned in the table.

**MIC value is tempting to speculate the isolate to be resistant. However, further confirmation by any other alternative method is advised.

*** According to CLSI guidelines, MIC <= 0.25 is considered as sensitive. But when isolate showed erythromycin resistance, it is speculated the isolates to be resistant to clindamycin as well (iMLSb phenotype)