Known mechanisms cannot account for at least one third of reduced susceptibility in a diverse collection of non-*aureus* staphylococci

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Running title: Antimicrobial resistance in non-*aureus* staphylococci

Abstract

Introduction: Non-*aureus* staphylococci (NAS) are implicated in many healthcare-acquired infections and an understanding of the genetics of antimicrobial resistance in NAS is important in relation to both clinical intervention and the role of NAS as a reservoir of resistance genes.

Gap statement: The burden of antimicrobial resistance in NAS, particularly to clinically relevant antimicrobials, is under recognised.

Methodology: We sourced 394 NAS isolates from clinical samples, healthy human volunteers, animals and type cultures and subjected them to agar dilution susceptibility testing against eight antimicrobials. Cefoxitin was used to screen for methicillin resistance in *S. aureus*, as it stimulates expression of *mecA*. We performed whole genome sequencing on 366 isolates and analysed these genotypically for the presence of genetic mechanisms responsible for the phenotypic levels of reduced antimicrobial susceptibility.

Results: We observed 175 sequenced isolates with a minimum inhibitory concentration (MIC) of at least 4 µg/ml to cefoxitin, of which 50% (87/175) did not harbour a known *mec* homologue. Eight clinical NAS isolates displayed high daptomycin MICs (>4 µg/ml), with no known mechanism identified. Differences in MICs against erythromycin were attributable to the presence of different resistance genes (*msrA* and *ermC*). In total, 49% (193 /394) of isolates displayed reduced susceptibility to three or more of the antimicrobials tested.

Conclusions: The widespread presence of reduced antimicrobial susceptibility in NAS is a concern, with an increased likelihood of (1) harder to treat infections caused directly by NAS, and (2) resistance genes being passed on to other bacteria via horizontal gene transfer, both of which have clinical implications for treatment and management of patients.

42 **Introduction**

43 The non-*aureus* staphylococci (NAS) represent an important source of nosocomial disease,
44 including prosthetic joint infection (PJI), infective endocarditis and infection in pre-term
45 babies (1). In the UK, over 215,000 joint replacements (hip, knee and shoulder) took place in
46 2016, with a year-on-year increase of 4% (2). Of these replacements, 1.5% require surgical
47 revision due to infection (2). These infections are most commonly caused by *Staphylococcus*
48 spp., and attributed to NAS in approximately 31% of cases across Europe (3). In our local
49 hospital, the Norfolk and Norwich University Hospital (NNUH), 50% of isolates identified in
50 suspected PJI are NAS.

51 In clinical microbiology, staphylococci are classified using the coagulase test, with coagulase
52 positive samples overwhelmingly identified as *S. aureus* and coagulase negative samples
53 grouped together under the term coagulase negative staphylococci (CoNS). CoNS is therefore
54 the term found in antimicrobial surveillance data. However, since coagulase negative *S.*
55 *aureus* strains exist (as do coagulase positive strains of other staphylococcal species), we use
56 the term "non-*aureus* staphylococci" (NAS) to encompass all staphylococci which are not *S.*
57 *aureus*, regardless of coagulase activity.

58 There is currently an intense focus upon the presence and spread of bacterial antimicrobial
59 resistance, typified in *S. aureus* by methicillin resistance (MRSA). While the body of literature
60 in antimicrobial resistance research is growing for staphylococci, NAS data remains eclipsed
61 by the focus on *S. aureus*. Few studies have investigated antimicrobial resistance (AMR) in
62 NAS. Those that have suggest 45% of NAS harbour methicillin resistance (4), and that NAS
63 may be resistant to a larger number of antimicrobial classes than *S. aureus* (4, 5).

64 An understanding of the genetics of AMR in NAS is important in relation to clinical
65 intervention upon diagnosis of PJI (and other infections) as well as understanding the role of

66 NAS as a reservoir of resistance genes. To address this, we assembled a collection of 394 NAS
67 from clinical samples, healthy human volunteers, animals and type cultures and assessed their
68 susceptibility to a range of antimicrobials. We also performed whole genome sequencing to
69 correlate mechanisms of resistance with minimum inhibitory concentrations (MICs).
70
71 **Materials & Methods**
72 *NAS collection*
73 Under NHS Research Ethics Committee approval, the Norwich Biorepository banks blood,
74 solid tissue and bacterial isolates from the NNUH and research institutes on the Norwich
75 Research Park, including the University of East Anglia (UEA), and makes these available to the
76 research community. This enabled us to assemble a collection of 380 NAS from a) clinical
77 specimens which were isolated from suspected NAS PJI infections (229, NNUH), b) healthy
78 human volunteers (114, UEA), and c) animal samples (33, UEA) with five having no source
79 recorded. An additional 14 strains of NAS from the National Collection of Type Cultures
80 (NCTC) were supplied by Public Health England.
81 Isolates were identified using MALDI-TOF (Bruker) to the species level (Table 1). All strains
82 were cultured overnight on TSA plates (Oxoid), checked for contamination and purified. Once
83 purified, the NAS collection was stored as glycerol stocks to be screened for their
84 antimicrobial susceptibility. *Staphylococcus aureus* NCTC 12973 was used as a control.
85
86 *Susceptibility testing*
87 To assay the entire NAS collection, five deep well 96-well microplates (VWR) were prepared
88 with 1 ml TSB (Oxoid) per well. Glycerol stocks were used to inoculate the corresponding well.
89 Per plate, one well was designated as a sterility control (TSB only) and one well was inoculated

90 with the *S. aureus* control. After inoculation, plates were sealed and incubated at 37 ^{o}C at
91 180 rpm for a minimum of 10 hours. The experimental design enabled 13.5% of the collection
92 to be tested in duplicate; MIC data was compared and then tabulated (Table S1).
93 Iso-sensitest agar (Oxoid) was prepared in 250 ml aliquots and autoclaved. Antimicrobial
94 stocks were added to obtain the desired final concentrations once the media had cooled to <
95 50 ^{o}C. For daptomycin, Ca^{2+} was also added at 50µg/ml. The agar antimicrobial mixture was
96 then poured into sterile rectangular plates (Fisher Scientific) and dried.
97 Per strain, a 1:10 dilution of overnight culture was transferred to a 96 well plate and the OD_{600}
98 was measured. An average OD_{600} was calculated for each column, which was then diluted to
99 approximately OD_{600} 0.6 to generate an inoculum plate for susceptibility testing.
100 Using a 96-pin multi-point inoculator (Denley), ~1 µl of inoculum per isolate was stamped
101 onto the agar containing antimicrobials, from the lowest concentration to the highest.
102 Between inoculum plates, the pins were washed in 70 % ethanol for 30 s and allowed to dry
103 before stamping on an antimicrobial-free plate to confirm sterility. Washes were also carried
104 out between antimicrobials using sterile water. All stamped plates were incubated at 37 ^{o}C.
105 Isolates found to have reduced susceptibility to daptomycin had their MICs determined for a
106 second time by spotting 10 µl of culture onto TSA plates containing various daptomycin
107 concentrations (supplemented with Ca^{2+} at 50 µg/ml). To increase *mecA* expression, 14
108 isolates which contained *mecA* but on initial testing showed susceptibility to cefoxitin (MIC <
109 4 µg/mL) were re-tested on Muller Hinton Agar with 3 % NaCl added alongside a further 6
110 isolates. Overnight cultures were diluted in PBS and 5 µl spots containing 10^4 cells were
111 spotted onto plates which were incubated at 35 ^{o}C.
112

113 Test MIC ranges were determined from British Society for Antimicrobial Chemotherapy
114 (BSAC) surveillance MIC data and EUCAST for coagulase negative staphylococci (CoNS) in 2016
115 (6). Data for daptomycin were taken from 2010. In µg/ml, the ranges tested were: daptomycin
116 0.25-2, erythromycin 0.125-256, gentamicin 0.016-64, rifampicin 0.004-0.064, teicoplanin
117 0.25-16, tetracycline 0.25-256 and vancomycin 1-4. No CoNS surveillance data were available
118 for cefoxitin, hence the test range of 0.25-4 µg/ml was based upon published work (7) .
119 Isolates were considered to have reduced susceptibility to the specified antibiotic if they
120 displayed the following MICs: $\geq$ 4 µg/mL (cefoxitin, teicoplanin, vancomycin); $\geq$ 2 µg/mL
121 (tetracycline, erythromycin); $\geq$ 1 µg/mL (gentamicin, daptomycin); $\geq$ 0.06 µg/mL (rifampicin).
122
123 *Statistical comparison of clinical and non-clinical isolates*
124 Using Prism (GraphPad, San Diego, USA, v 5.04), a Mann-Whitney test was performed (non-
125 parametric test, two-tailed with Gaussian approximation) to compare between the MIC of
126 clinical and non-clinical isolates. Statistical significance was given to a p value < 0.05.
127
128 *DNA extraction and sequencing*
129 Overnight cultures derived from single colonies were pelleted and resuspended in lysis buffer
130 (Qiagen), transferred to 2 ml lysis matrix B tubes (MPBio) and subjected to bead beating for
131 15 min at 30 Hz (Tissuelyser II, Qiagen) with RNAse A added. DNA was extracted according to
132 the QiaCube HT protocol with an additional 30 min incubation at 65 ^{o}C after proteinase K
133 addition and eluted into Tris-10mM HCL.
134
135 Libraries for sequencing were prepared using the Nextera XT DNA Library Prep Protocol and
136 sequenced on the Illumina MiSeq or NextSeq with a loading concentration of 1.8 picomolar.

137

138 *Genome analysis*

139 The raw reads were subject to FastQC quality control (8), adapters were trimmed using
140 Trimmomatic [v 0.39] (9) using the supplied NexteraXT adapter sequences. In some cases,
141 read normalisation was performed using BBNorm [v35.85] (10) to remove low coverage
142 contamination. The lowest coverage cutoff level parameter used was dependent on the total
143 coverage of the sequence since some sequencing runs had a high difference in coverage level
144 across the run. Finally, reads were concatenated if they originated from Illumina NextSeq
145 since this platform produces eight reads per sample, four forward and four reverse. A total
146 of 364 samples passed QC and were suitable for downstream analysis. These reads were used
147 as described below. Sequences are available from the European Nucleotide Archive, under
148 project PRJEB31403.

149 To determine which antimicrobial resistance genes and associated individual mutations were
150 present in each of our 364 NAS genomes, reference gene sequences were downloaded from
151 CARD v2.0.0 (11) and used as input to ARIBA v2.13.2 (12) which generates local assemblies
152 from sequence reads and reports back which reference genes (and individual mutations) are
153 identified, with a minimum percent identity cut off at 90% (Table S5). For genes where 'partial'
154 or 'interrupted' was reported, this was not considered sufficient evidence for intact gene
155 presence. The tabulated results were evaluated for gene and mutation presence/absence
156 relative to MIC per antimicrobial. Twelve NCTC sequences were downloaded as genome
157 assemblies from the European Nucleotide Archive (accessions: SAMEA4364213;
158 SAMEA4364214; SAMEA4384234; SAMEA4384058; SAMEA4384237; SAMEA4384064;
159 SAMEA4412661; SAMEA4384059; SAMEA4384235; SAMEA4384060; SAMEA4384339;
160 SAMEA4384403) and analysed by ABRIcate v0.9.7 (13) using CARD v2.0.0 (11) as the reference

161 database with a minimum DNA coverage of 90%. NCTC 13831 and 13837 sequence data was
162 not available at time of sequencing and therefore these isolates were sequenced as described
163 above for the main NAS collection. Protein level conservation was assessed using BLAST
164 v2.10.1 against the NCBI AMR database. Hits were recorded for greater than 40% identity at
165 the protein level over 80% of the query and subject sequence.
166
167 **Results & Discussion**
168 Our NAS collection comprised over 30 species of *Staphyloccocus*, including at least 10 isolates
169 of *S. epidermidis*, *S. capitis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *S. simulans* and *S.*
170 *warneri* (Table 1). Isolates were collected over a four-year period from 2013 to 2016.
171 The range of antimicrobials tested were selected based upon clinical relevance and availability
172 (Table S1). Observed MIC distributions per antimicrobial are shown in Figure 1.
173
174 *Cefoxitin screening does not correlate with* mecA *presence in clinically relevant NAS*
175 Cefoxitin is used to screen for methicillin resistance in *S. aureus* as it induces *mecA* expression.
176 However, while methicillin resistant *S. aureus* (MRSA) have a high public profile, much less is
177 known about methicillin resistant NAS (MRNAS). EUCAST guidelines state that for MRSA
178 "cefoxitin is a very sensitive and specific marker of *mecA/mecC*-mediated methicillin
179 resistance including in heterogeneous expressing strains and is the agent of choice" (14). In
180 this collection, we found 194/394 (49%) displayed reduced susceptibility to cefoxitin with
181 MICs $\geq$ 4 µg/ml (Table S1). The vast majority of these isolates were from clinical samples (FOX
182 Figure 1, Table S1) but analysis at the nucleotide level (Table S2 and S3) indicated that only
183 88 out of 175 (50 %) sequenced isolates with an MIC $\geq$ 4µg/ml harboured a known *mecA.*
184 MecA is extremely well-conserved and searching at the protein level yielded the same results

185 (Table S4). Other *mec* elements were also identified (e.g. *mecC*, *mecI* and *mecR1*) but only
186 ever in addition to *mecA*. Breaking this down by species, 20/21 *S. saprophyticus* isolates with
187 cefoxitin MIC ≥ 4µg/ml harboured no *mecA* (Figure 2). No *mecA* was detected in eleven
188 species with high cefoxitin MICs and for *S. hominis*, *S. warneri* and *S. haemolyticus,* the
189 percentage of the population that exhibited MIC ≥ 4µg/ml with no *mecA* was between 28 and
190 67% (Figure 2). Our results support cefoxitin as a good indicator of *mecA* presence in *S.*
191 *epidermidis* (7), but suggest that it performs poorly in the less common, but still clinically
192 relevant NAS. In addition, we observed 14 cases where the presence of *mecA* did not result
193 in an MIC ≥ 4µg/ml. These were re-tested (alongside 6 others) under conditions designed to
194 encourage *mecA* expression and resulted in increased MICs in all isolates, however 7/20
195 remained <4 ug/mL (Table S1).
196
197 *Reduced susceptibility only partly explained by known mechanisms*
198 When exposed to gentamicin and tetracycline, isolates could broadly be divided into two
199 populations, displaying susceptible or reduced susceptibility phenotypes (Figure 1 GEN and
200 TET). In isolates displaying an MIC ≥1 µg/ml for gentamicin, 49/130 isolates harboured *aac(6')-*
201 *Ie-aph(2")-Ia* (Table 2) which is associated with gentamicin resistance in *Enterococcus* (15, 16)
202 but has also been observed in *Staphylococcus* (17, 18). A total of 12 isolates had a match for
203 *aph(3')IIIa*, but only five of them were associated with reduced susceptibility.
204 Six isolates that contained *aac(6')-Ie-aph(2")-Ia* displayed susceptible MICs, making them the
205 equivalent of major errors (MEs) in public health terms, as the isolates were genotypically
206 resistant but phenotypically susceptible (19). Accordingly, the 81/130 isolates with reduced
207 susceptibility (≥1 µg/ml) that harboured no *aac(6')-Ie-aph(2")-Ia* represented the equivalent
208 of very major errors (VMEs) as they were genotypically susceptible but phenotypically

209 resistant (19). This is highly suggestive of novel mechanisms of resistance and was a feature
210 of other antimicrobials tested (Figure 3).
211 To identify whether efflux pumps might play a role these phenotypes, we assessed the ARIBA
212 output for the staphylococcal-specific *norABC, mgrA, mepR* and *qac* genes (20). In the
213 sequenced NAS collection 168/378 (44.4 %) contained *norA*, however of these less than two
214 thirds (75/130) had reduced susceptibility to gentamicin.
215 According to the Comprehensive Antimicrobial Resistance Database [CARD] (11), *tetK* is by far
216 the most common tetracycline resistance mechanism in *S. aureus* and *S. epidermidis* (10-
217 20%), followed by *tetL* (<1%) and *tetM* (<1%). This was borne out in our NAS collection, where
218 48/148 (32.4 %) isolates with MICs ≥ 2 µg/ml of tetracycline contained *tetK*, as compared to
219 5/223 (2.2 %) with MICs below 2 µg/ml. One animal isolate with an MIC of 16 µg/ml carried
220 *tetL* and one clinical isolate with an MIC of 64 µg/ml carried *tetM*; neither had any other
221 tetracycline resistance genes. Again, this demonstrated that 98/148 isolates displayed a
222 reduced susceptibility phenotype that did not associate with a known resistance determinant,
223 indicative of uncharacterised resistance mechanisms.
224
225 The distribution of erythromycin phenotypes was more complex. With this antimicrobial, we
226 observed both susceptible isolates and those with reduced susceptibility, but the latter
227 appeared to consist of two populations, one with MICs between ≥2-256 µg/ml and one with
228 MICs ≥512 µg/ml (Figure 1 ERY). We had sequence data available from 135 of the ≥2-256
229 µg/ml population and 66 of the ≥512 µg/ml population, and identified the presence of a
230 resistance gene (*ermA, ermC, msrA)* in 65.9 % (89/135) of the ≥2-256 µg/ml population and
231 55/66 (83.3 %) of the ≥512 µg/ml population (Table 2). Our results indicated that the presence
232 of *ermC* rather than *msrA* was the major cause of MICs exceeding 256 µg/ml. Although rare

233 (n=4), harbouring both genes resulted in an MIC ≥ 512 µg/ml in three cases and 128 µg/ml in
234 the other. In isolates with an MIC of ≥ 512 µg/ml, *qac* was identified 36 times. In 23 of these
235 cases *ermC* was also present; *qac* was only found twice with no other known erythromycin
236 resistance mechanisms present. A total of 101 isolates with an MIC ≥2 µg/ did not contain
237 *qac.*
238
239 For daptomycin, approximately half the collection displayed reduced susceptibility (MIC ≥ 1
240 µg/ml, Figure 1 DAP and Table S1). A small subset, comprising eight isolates from clinical
241 samples only, displayed MICs ≥ 4 µg/ml; such high MICs to daptomycin have not been
242 previously reported, according to the European Committee on Antimicrobial Susceptibility
243 Testing (EUCAST) and The British Society for Antimicrobial Chemotherapy (BSAC) surveillance
244 data. These MICs were repeated a second time and confirmed. This is concerning given that
245 daptomycin is a current therapeutic choice for treating soft tissue infections caused by NAS
246 (21). Seven of these isolates were sequenced and our ARIBA analysis (Table 3) indicated only
247 a single *S. epidermidis* isolate contained genes implicated in daptomycin resistance: *gshF* and
248 *liaFRS* with an MIC of 1 µg/ml, the remaining 169 isolates with an ≥1 µg/ml MIC did not
249 harbour any of these genes. Several mutations or genes are associated with daptomycin
250 resistance in *S. aureus* (including *mprF*, and SNPs in *rpoC*) but none of these were identified
251 in the NAS collection (6, 22). More recently, SNPs in *walK* have also been associated with
252 daptomycin resistance in *S. aureus* and *S. epidermidis* (23). The three *S. aureus* SNPs are
253 present in CARD and were not identified in our collection. The V500F mutation from *S.*
254 *epidermidis* (23) was also not identified in our *S. epidermidis* with DAP MICs ≥ 4 µg/ml. Whilst
255 *walK* was identified by protein BLAST as present across the NAS collection (as expected for an
256 essential gene (24)), sequence variation was observed at the protein level which prevents

257 SNPs observed in *S. aureus* or *S. epidermidis* being extrapolated to NAS. We therefore
258 conclude that there are potentially novel daptomycin resistance mechanisms present in these
259 strains.
260
261 *Resistance to vancomycin found in clinical samples*
262 Vancomycin is a treatment option in prosthetic joint infection, and 94 % of isolates had an
263 MIC below 4 µg/ml (Figure 1 VAN). However, of the 24 isolates with reduced susceptibility,
264 22 (92 %) came from clinical samples and only 2/24 were found in healthy volunteers. This is
265 indicative of a wider trend, where isolates associated with clinical samples had significantly
266 higher MICs ($p < 0.005$) than non-clinical isolates for cefoxitin, erythromycin, gentamicin,
267 tetracycline, daptomycin and vancomycin (Figure S1). Given the importance of NAS in
268 nosocomial infections, this is a worrying prospect both in terms of what is presenting in the
269 clinic and also the possibility of AMR gene transfer into organisms more capable of causing
270 infection, including *S. aureus*. In addition, no known mechanisms of resistance were identified
271 for vancomycin, rifampicin or teicoplanin (Figure 3 and Table S3).
272
273 *Over half of the NAS collection displayed susceptibility to multiple antimicrobials*
274 Out of the all the isolates tested, 48 % of (192/394) had reduced susceptibility to three or
275 more antimicrobials. Twenty-five isolates had reduced susceptibility to six antimicrobials, and
276 three isolates had reduced susceptibility to seven antimicrobials; of these 24/25 and 3/3 were
277 isolated from clinical samples (Table S1). The implications of these are difficult to treat
278 infections and potentially a large reservoir of staphylococcal resistance genes within the
279 patient under antimicrobial treatment.
280

281 *Animal isolates have similar MIC distributions to human isolates*

282 It is generally acknowledged that the presence of reduced susceptibility in microorganisms
283 isolated from animals can impact upon public health if those organisms also cause infection
284 in humans (25, 26). In our collection there were 40 NAS isolated from animals (7 NCTC strains),
285 of which we obtained genome sequences from 23. Although in much fewer numbers than the
286 human isolates in the collection, the animal isolates displayed very similar MIC distributions
287 and harboured corresponding genetic mechanisms. This does not rule out the possibility that
288 animals could be a reservoir of AMR for staphylococci.

289

290 **Conclusion**

291 Genome analysis of isolates displaying MICs to cefoxitin of ≥ 4 µg/ml indicated that
292 approximately half harboured the *mecA* element. The absence of *mecA* from the other half
293 suggests that other mechanisms are likely present. This was apparent across many of the
294 antimicrobials tested as between 0 and 65 % of phenotypic resistance in clinical isolates could
295 be attributed to known resistance mechanisms. The remaining 35-100 % suggests that there
296 are potentially numerous unknown mechanisms underpinning NAS resistance, which warrant
297 further investigation.

298

299 **Acknowledgements**

300 We thank David Livermore and Iain McNamara for productive discussions on antimicrobial
301 usage.

302

303 **Funding**

304 This work was funded by the Orthopaedics Trust (registered charity 1110248).

305

306 **Transparency declarations**

307 None to declare.

308

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393

394

395

396

397 **Figure legends**

398 Figure 1. Antibiotic susceptibility of NAS collection

399 MIC distributions for 394 NAS isolates grown in the presence of antimicrobials. FOX cefoxitin,

400 GEN gentamicin, TET tetracycline, ERY erythromycin, DAP daptomycin, VAN vancomycin, RIF

401 rifampicin and TEC teicoplanin.

402

403 Figure 2. Presence of *mecA* in relation to high cefoxitin MIC.

404 Per staphylococcal species, bars display the total number of sequenced isolates found to have

405 a cefoxitin MIC of ≥4 µg/ml, in relation to the presence (solid green) and absence (blue

406 outline) of *mecA*.

407

408 Figure 3. Mechanisms of resistance

409 Percentage of known genetic mechanisms identified in sequenced NAS isolates with reduced

410 susceptibility. Number of isolates with reduced susceptibility per antimicrobial given in

411 parentheses. Known mechanisms found per antimicrobial are shown in italics. FOX cefoxitin,

412 GEN gentamicin, TET tetracycline, ERY erythromycin, DAP daptomycin, VAN vancomycin, RIF

413 rifampicin and TEC teicoplanin.

414

415

416

417 Table 1

418 Frequency of non-*aureus* staphylococcal species in the study collection

Staphylococcus auricularis	1
Staphylococcus capitis	20
Staphylococcus caprae	2
Staphylococcus carnosus	2
Staphylococcus chromogenes	3
Staphylococcus cohnii	1
Staphylococcus condimenti	1
Staphylococcus devriesei	1
Staphylococcus epidermidis	191
Staphylococcus equorum	1
Staphylococcus haemolyticus	43
Staphylococcus hominis	45
Staphylococcus jettensis	1
Staphylococcus lugdunensis	7
Staphylococcus massiliensis	1
Staphylococcus microti	1
Staphylococcus muscae	1
Staphylococcus nepalensis	1
Staphylococcus pasteuri	4
Staphylococcus petrasii	1
Staphylococcus pettenkferi	1
Staphylococcus piscifermentans	1
Staphylococcus rostri	1
Staphylococcus saprophyticus	22
Staphylococcus sciuri	5
Staphylococcus simiae	1
Staphylococcus simulans	10
Staphylococcus sp [1]	1
Staphylococcus stepanovicii	1
Staphylococcus succinus	1
Staphylococcus vitulinus	3
Staphylococcus warneri	18
Staphylococcus xylosus	1

419

420 *Species designated by MALDI-TOF (Bruker)

421

422 Table 2 Genetic mechanisms identified using ARIBA/ABRIcate and the CARD database
423 compared to the MIC data (of sequenced isolates only, partial and interrupted sequences are
424 not included, see Table S2)
425

Antimicrobial	*Mechanism (Accession No.)*	*No. isolates above breakpoint MIC (≥2 µg/ml)*		*No. isolates below breakpoint*
TET	*tetK* (NC_013452)	48/148		5/223
	tetL (M11036.0)	1/148		0/223
	tetM (AM180355)	1/148		0/223
	All*	**50/**148 **(33.8%)**		**5/223 (2.2%)**
		No. isolates above breakpoint MIC (≥1 µg/ml)		
GEN	*aac(6')-Ie-aph(2")-Ia* (NC_005024)	49/130		6/246
	aph(3)IIIa (CP004067)	5/130		7/246
	All*	**49/130 (37.7 %)**		**12/246 (4.9 %)**
		MIC ≥512 µg/ml	*MIC ≥2 - 256 µg/ml*	
ERY	*msrA* (NC_022598.1)	6/66	75/135	9/173
	ermC (M12730)	42/66	13/135	9/173
	ermA (NC_009632)	6/66	1/135	0/173
	emeA (AB091338)	1/66	0/135	0/173
	All*	**46/66 (69.7 %)**	**88/135 (65.2 %)**	**18/173 (10.4 %)**

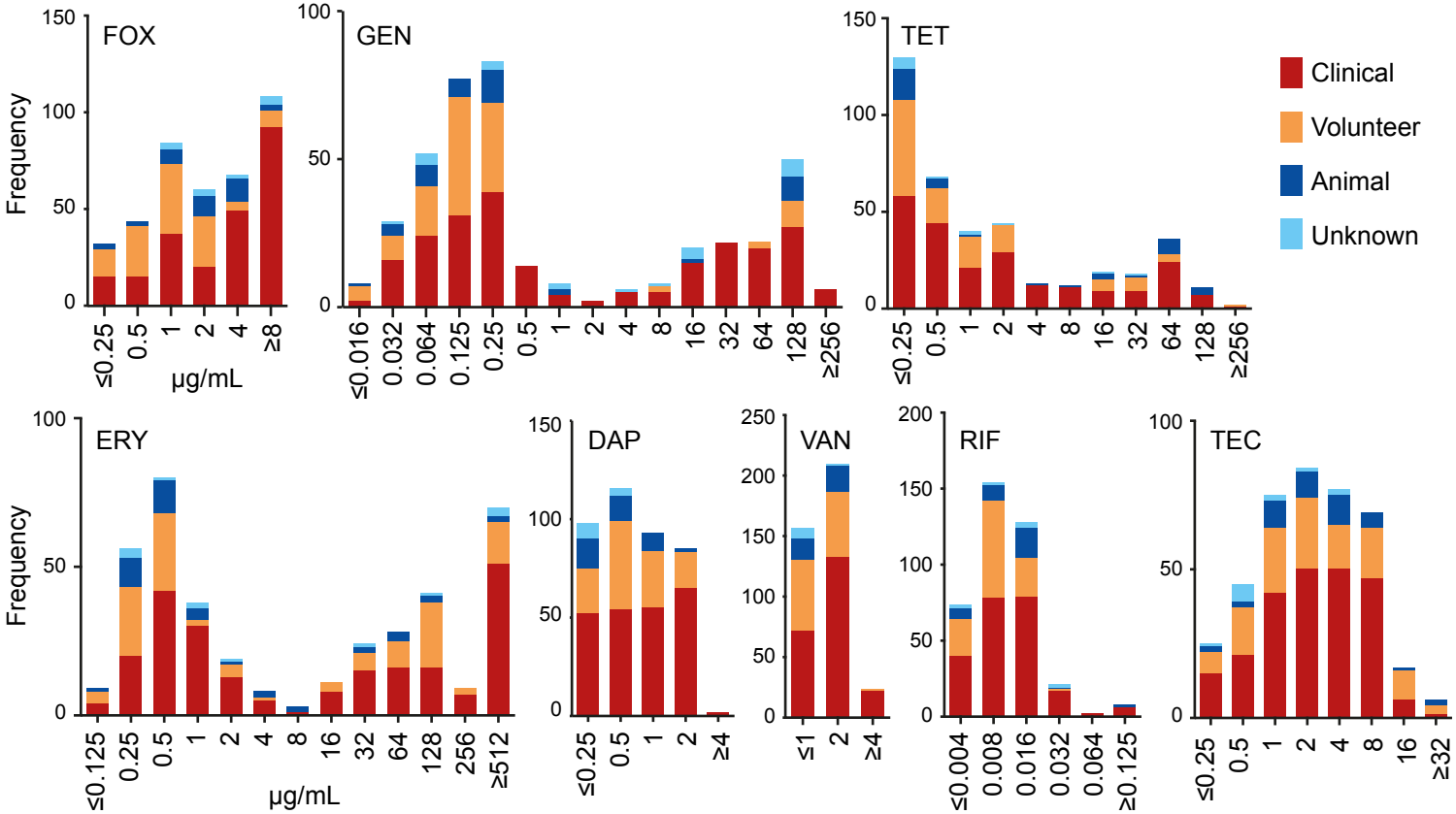
426
427 *Some isolates harboured multiple resistance genes. TET tetracycline, GEN gentamicin, ERY
428 erythromycin.
429

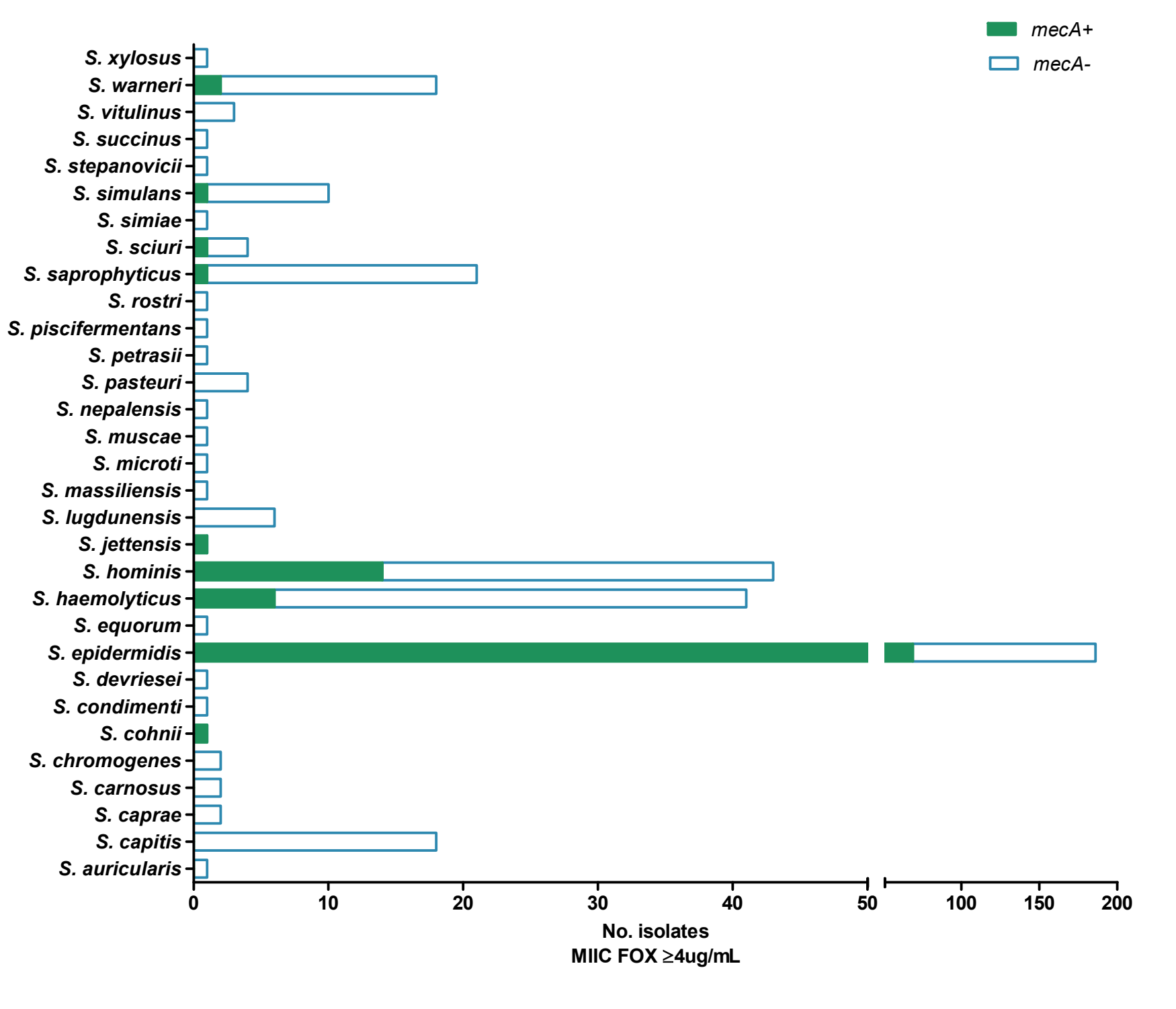
430

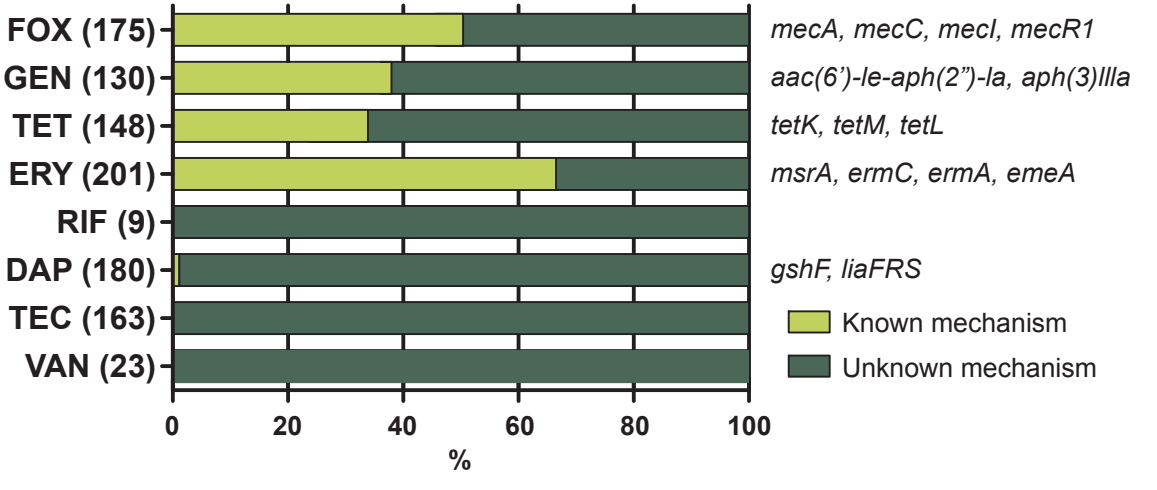
431 Supplementary data

432 Table S1. MIC characterisation of the NAS collection

433 MIC data for all isolates tested; all MIC are shown in µg/ml; FOX cefoxitin, GEN gentamicin,
434 TET tetracycline, ERY erythromycin, DAP daptomycin, VAN vancomycin, RIF rifampicin and
435 TEC teicoplanin.
436
437 Table S2. ARIBA characterisation of all sequenced NAS isolates, grouped by relevant
438 antimicrobial.
439
440 Table S3. Summary of ARIBA/ABRIcate match data for all sequenced isolates.
441
442 Table S4. Comparison of gene presence (using ARIBA) and protein BLAST presence. Data
443 includes all genes identified in the CARD database.
444
445 Table S5. Full list of all reference genes/mutations from the CARD database analysed for
446 presence/absence.
447
448 Figure S1. MICs in clinical and non-clinical isolates
449 Box and whisker plot of MIC distribution per antimicrobial for clinical (orange, CS) and non-
450 clinical (yellow, NC) isolates. Thick black bar indicates the median MIC which resided within
451 the Interquartile Range. The extreme lines are represented as dotted lines and indicate the
452 data outside the upper (75%) and lower (25%) quartiles, open circles represent potential
453 outliers. Levels of significance from Mann-Whitney U test denoted by **** ($p<0.0001$), ***
454 ($p<0.005$), ** ($p=0.001$) and * ($p=0.01$).
455
456


FOX
GEN
TET
ERY
DAP
VAN
RIF
TEC
Frequency
μg/mL
Clinical
Volunteer
Animal
Unknown
≤0.25
0.5
1
2
4
≥8
≤0.016
0.032
0.064
0.125
0.25
8
16
32
64
128
≥256
≤0.125
256
≥512
≥4
≤1
≤0.004
0.008
0.016
0.032
0.064
≥0.125
≥32


mecA+
mecA-
S. xylosus
S. warneri
S. vitulinus
S. succinus
S. stepanovicii
S. simulans
S. simiae
S. sciuri
S. saprophyticus
S. rostri
S. piscifermentans
S. petrasii
S. pasteuri
S. nepalensis
S. muscae
S. microti
S. massiliensis
S. lugdunensis
S. jettensis
S. hominis
S. haemolyticus
S. equorum
S. epidermidis
S. devriesei
S. condimenti
S. cohnii
S. chromogenes
S. carnosus
S. caprae
S. capitis
S. auricularis
0
10
20
30
40
50
100
150
200
No. isolates
MIIC FOX ≥4ug/mL


FOX (175)
GEN (130)
TET (148)
ERY (201)
RIF (9)
DAP (180)
TEC (163)
VAN (23)
mecA, mecC, mecI, mecR1
aac(6')-Ie-aph(2")-Ia, aph(3)IIIa
tetK, tetM, tetL
msrA, ermC, ermA, emeA
gshF, liaFRS
Known mechanism
Unknown mechanism
0
20
40
60
80
100
%