1 **Known mechanisms account for less than half of antimicrobial resistance in a diverse**
2 **collection of non-*aureus* staphylococci.**

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

19 Abstract

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

24 Gap statement: The burden of antimicrobial resistance in NAS, particularly to clinically
25 relevant antimicrobials, is understudied.

26 Methodology: We sourced 394 NAS isolates from clinical samples, healthy human
27 volunteers, animals and type cultures and subjected them to agar dilution susceptibility
28 testing against eight antimicrobials. We performed whole genome sequencing on 316
29 isolates and analysed these genotypically for the presence of genetic mechanisms
30 responsible for the phenotypic levels of reduced antimicrobial susceptibility.

31 Results: Cefoxitin is used to screen for methicillin resistance in *S. aureus*, as it stimulates
32 expression of *mecA*. We observed 174 isolates with an MIC of at least 4 µg/ml to cefoxitin,
33 of which sequencing revealed 47.6% (80/168) did not harbour a known *mec* homologue.
34 Seven clinical NAS isolates displayed high daptomycin minimum inhibitory concentrations
35 (MICs) (>4 µg/ml), with no known mechanism identified. Differences in MICs against
36 erythromycin were attributable to the presence of different resistance genes (*msrA* and
37 *ermC*). In total, 49% (187/394) of isolates displayed reduced susceptibility to three or more
38 of the antimicrobials tested.

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

43 Introduction

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

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

59 There is currently an intense focus upon the presence and spread of bacterial antimicrobial
60 resistance, typified in *S. aureus* by methicillin resistance (MRSA). While the body of
61 literature in antimicrobial resistance research is growing for staphylococci, NAS data
62 remains eclipsed by the focus on *S. aureus*. Few studies have investigated antimicrobial
63 resistance (AMR) in NAS. Those that have suggest 45% of NAS harbour methicillin resistance
64 (4), and that NAS may be resistant to a larger number of antimicrobial classes than *S. aureus*
65 (4, 5).

An understanding of the genetics of AMR in NAS is important in relation to clinical intervention upon diagnosis of PJI (and other infections) as well as understanding the role of NAS as a reservoir of resistance genes. To address this, we assembled a collection of 400 NAS from clinical samples, healthy human volunteers, animals and type cultures and assessed their susceptibility to a range of antimicrobials. We also performed whole genome sequencing to correlate mechanisms of resistance with minimum inhibitory concentrations (MICs).

73

74 **Materials & Methods**

75 *NAS collection*

Under NHS Research Ethics Committee approval, the Norwich Biorepository banks blood, solid tissue and bacterial isolates from the NNUH and research institutes on the Norwich Research Park, including the University of East Anglia, and makes these available to the research community. This enabled us to assemble a collection of 380 NAS from a) clinical specimens which were isolated from suspected NAS PJI infections (229, NNUH), b) healthy human volunteers (114, UEA), and c) animal samples (32, UEA) with five having no source recorded. An additional 14 strains of NAS from the National Collection of Type Cultures (NCTC) were supplied by Public Health England.

Isolates were identified using MALDI-TOF (Bruker) to the species level (Table 1). All strains were cultured overnight on TSA plates (Oxoid), checked for contamination and purified. Once purified, the NAS collection was stored as glycerol stocks to be screened for their antimicrobial susceptibility. *Staphylococcus aureus* NCTC 12973 was used as a control.

88

89 *Susceptibility testing*

90 To assay the entire NAS collection, five deep well 96-well microplates (VWR) were prepared
 91 with 1 ml TSB (Oxoid) per well. Glycerol stocks were used to inoculate the corresponding
 92 well. Per plate, one well was designated as a sterility control (TSB only) and one well was
 93 inoculated with the *S. aureus* control. After inoculation, plates were sealed and incubated
 94 at 37 °C at 180 rpm for a minimum of 10 hours. The experimental design enabled 13.5% of
 95 the collection to be tested in duplicate; so MIC data was compared and then tabulated, MIC
 96 data is given in Table S1.

97 Iso-sensitest agar (Oxoid) was prepared in 250 ml aliquots and autoclaved. Antimicrobial
 98 stocks were added to obtain the desired final concentrations once the media had cooled to
 99 < 50 °C. For daptomycin, Ca²⁺ was also added at 50µg/ml. The agar antimicrobial mixture
 100 was then poured into sterile rectangular plates (Fisher Scientific) and dried.

101 Per strain, a 1:10 dilution of overnight culture was transferred to a 96 well plate and the
 102 OD₆₀₀ was measured. An average OD₆₀₀ was calculated for each column, which was then
 103 diluted to approximately OD₆₀₀ 0.6 to generate an inoculum plate for susceptibility testing.

104 Using a 96-pin multi-point inoculator (Denley), ~1 µl of inoculum per isolate was stamped
 105 onto the agar containing antimicrobials, from the lowest concentration to the highest.

106 Between inoculum plates, the pins were washed in 70 % ethanol for 30 s and allowed to dry
 107 before stamping on an antimicrobial-free plate to confirm sterility. Washes were also
 108 carried out between antimicrobials using sterile water. All stamped plates were incubated at
 109 37 °C.

110 Isolates found to have reduced susceptibility to daptomycin had their MICs determined for a
 111 second time by spotting 10 µl of culture onto TSA plates containing various daptomycin
 112 concentrations (supplemented with Ca²⁺ at 50 µg/ml).

Test MIC ranges were determined from British Society for Antimicrobial Chemotherapy (BSAC) surveillance MIC data for coagulase negative staphylococci (CoNS) in 2016 (6). Data for daptomycin were taken from 2010. In µg/ml, the ranges tested were: daptomycin 0.25-4, erythromycin 0.125-256, gentamicin 0.016-64, rifampicin 0.004-0.064, teicoplanin 0.25-16, tetracycline 0.25-256 and vancomycin 1-4. No CoNS surveillance data were available for cefoxitin, hence the test range of 0.25-4 µg/ml was based upon published work (7) .

Statistical comparison of clinical and non-clinical isolates

Using Prism (GraphPad, San Diego, USA, v 5.04), a Mann-Whitney test was performed (non-parametric test, two-tailed with Gaussian approximation) to compare between the MIC of clinical and non-clinical isolates. Statistical significance was given to a p value < 0.05.

DNA extraction and sequencing

Overnight cultures derived from single colonies were pelleted and resuspended in lysis buffer (Qiagen), transferred to 2 ml lysis matrix B tubes (MPBio) and subjected to bead beating for 15 min at 30 Hz (Tissuelyser II, Qiagen) with RNase A added. DNA was extracted according to the QiaCube HT protocol with an additional 30 min incubation at 65 °C after proteinase K addition and eluted into Tris-10mM HCL.

Libraries for sequencing were prepared using the Nextera XT DNA Library Prep Protocol and sequenced on the Illumina MiSeq or NextSeq with a loading concentration of 1.8 picomolar.

Genome analysis

The raw reads were subject to FastQC quality control (8), adapters were trimmed using Trimmomatic [v 0.39] (9) using the supplied NexteraXT adapter sequences. In some cases, read normalisation was performed using BBNorm [v35.85] (10) to remove low coverage contamination. The lowest coverage cutoff level parameter used was dependent on the total coverage of the sequence since some sequencing runs had a high difference in coverage level across the run. Finally, reads were concatenated if they originated from Illumina NextSeq since this platform produces eight reads per sample, four forward and four reverse. A total of 366 samples sequenced for this project passed QC and were suitable for downstream analysis. These reads were used as described below. Sequences are available from the European Nucleotide Archive, under project PRJEB31403.

To determine which antimicrobial resistance genes were present in each of our 364 NAS genomes, reference gene sequences were downloaded from CARD v (11) and used as input to ARIBA v2.13.2 (12) which generates local assemblies from sequence reads and reports back which reference genes are identified, with a minimum percent identity cut off at 90%. The tabulated results were evaluated for gene/mutation presence/absence relative to MIC per antimicrobial. Twelve of the NCTC strains, for which sequence data were already available, were analysed by ABRicate v0.9.7 (13) using CARD v2.0.0 (11) as the reference database with a minimum DNA coverage of 90%. Protein level conservation was assessed using BLAST v2.10.1 against the NCBI AMR database. Hits were recorded for greater than 40% identity at the protein level over 80% of the query and subject sequence.

Results & Discussion

Our NAS collection comprised over 30 species of *Staphylococcus*, including 10 or more isolates of *S. epidermidis*, *S. capitis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *S. simulans* and *S. warneri* (Table 1). Isolates were collected over a four-year period from 2013 to 2016.

The range of antimicrobials tested were selected based upon clinical relevance and availability (Table S1). Observed MIC distributions per antimicrobial are shown in Figure 1.

Cefoxitin screening does not correlate with mecA presence in clinically relevant NAS

Cefoxitin is used to screen for methicillin resistance in *S. aureus* as it induces *mecA* expression. However, while methicillin resistant *S. aureus* (MRSA) have a high public profile, much less is known about methicillin resistant NAS (MRNAS). EUCAST guidelines state that for MRSA “cefoxitin is a very sensitive and specific marker of *mecA/mecC*-mediated methicillin resistance including in heterogeneous expressing strains and is the agent of choice” (14). In this collection, we found 174/394 (44%) displayed reduced susceptibility to cefoxitin with MICs ≥ 4 μ g/ml (Table S1). The vast majority of these isolates were from clinical samples (Figure 1) but analysis at the nucleotide level (Table S2 and S3) indicated that only 80/168 (47.6 %) with an MIC ≥ 4 μ g/ml harboured a known *mecA*. *MecA* is extremely well-conserved and searching at the protein level yielded the same results (Table S4). Other *mec* elements were also identified (e.g. *mecC*, *mecI* and *mecR1*) but only ever in addition to *mecA*. No *mecA* was detected in twelve species with high cefoxitin MICs and for *S. haemolyticus*, *S. warneri*, *S. sciuri* and *S. simulans* the percentage of the population that exhibited MIC ≥ 4 μ g/ml with no *mecA* was found between 58 and 88.9% (Figure 2). Conversely, presence of *mecA* did not result in a high cefoxitin MIC in multiple isolates of *S. hominis*, *S. haemolyticus* and *S. epidermidis*. Our results therefore do not support cefoxitin

as a universally good indicator of *mecA* presence in *S. epidermidis* (7), and suggest that it performs poorly in the less common, but still clinically relevant NAS.

Clinical resistance only partly explained by known mechanisms

When exposed to gentamicin and tetracycline, isolates could broadly be divided into two populations, displaying susceptible or reduced susceptibility phenotypes (Figure 1 GEN and TET). In isolates with an MIC ≥ 1 $\mu\text{g/ml}$ gentamicin, 49/130 harboured *aac(6')-le-aph(2'')-la* (Table 2) which is associated with gentamicin resistance in *Enterococcus* (15, 16) but has also been observed in *Staphylococcus* (17, 18). A total of 12 isolates had a match for *aph(3')IIIa*, but only five of them were associated with reduced susceptibility.

Six isolates that contained *aac(6')-le-aph(2'')-la* displayed susceptible MICs, making them the equivalent of major errors (MEs) in public health terms, as the isolates were genotypically resistant but phenotypically susceptible (19). Accordingly, the 75/130 isolates with reduced susceptibility (≥ 1 $\mu\text{g/ml}$) that harboured no *aac(6')-le-aph(2'')-la* represented the equivalent of very major errors (VMEs) as they were genotypically susceptible but phenotypically resistant (19). This is highly suggestive of novel mechanisms of resistance and was a feature of other antimicrobials tested (Figure S2).

To identify whether efflux pumps might play a role these phenotypes, we assessed the ARIBA output for the staphylococcal-specific *norABC*, *mgrA*, *mepR* and *qac* genes (20). In the sequenced NAS collection 168/378 (44.4 %) contained *norA*, however of these less than two thirds had reduced susceptibility to gentamicin.

According to the Comprehensive Antimicrobial Resistance Database [CARD] (11), *tetK* is by far the most common tetracycline resistance mechanism in *S. aureus* and *S. epidermidis* (10-20%), followed by *tetL* (<1%) and *tetM* (<1%). This was borne out in our NAS collection,

where 48/148 (32.4 %) isolates with MICs ≥ 2 $\mu\text{g/ml}$ of tetracycline contained *tetK*, as compared to 5/223 (2.2 %) with MICs below 2 $\mu\text{g/ml}$. One animal isolate with an MIC of 16 $\mu\text{g/ml}$ carried *tetL* and one clinical isolate with an MIC of 64 $\mu\text{g/ml}$ carried *tetM*; neither had any other tetracycline resistance genes. Again, this demonstrated that 98/148 isolates displayed a reduced susceptibility phenotype that did not associate with a known resistance determinant, indicative of uncharacterised resistance mechanisms.

The distribution of erythromycin phenotypes was more complex. With this antimicrobial, we observed both susceptible isolates and those with reduced susceptibility, but the latter appeared to consist of two populations, one with MICs between 2 and 256 $\mu\text{g/ml}$ and one with MICs ≥ 512 $\mu\text{g/ml}$ (Figure 1 ERY). We had sequence data available from 135 of the 2-256 $\mu\text{g/ml}$ population and 66 of the ≥ 512 $\mu\text{g/ml}$ population, and identified the presence of a resistance gene (*ermA*, *ermC*, *msrA*) in 65 % (88/135) of the 2-256 $\mu\text{g/ml}$ population and 52/66 (79 %) of the ≥ 512 $\mu\text{g/ml}$ population (Table 2). Our results indicated that the presence of *ermC* rather than *msrA* was the major cause of MICs exceeding 256 $\mu\text{g/ml}$. In the total sequenced NAS collection *qac* was observed 100 times, with 64 isolates having a reduced susceptibility to erythromycin (2-256 $\mu\text{g/ml}$ population). Seven isolates had no other known mechanisms. *qac* was identified 36 with an MIC of ≥ 512 $\mu\text{g/ml}$ and in 23 of these cases *ermC* was also present, only twice was *qac* found with no other known erythromycin resistance mechanisms. A total of 101 isolates with an MIC ≥ 2 $\mu\text{g/ml}$ did not contain *qac*.

For daptomycin, approximately half the collection displayed reduced susceptibility (MIC ≥ 1 $\mu\text{g/ml}$, Figure 1 DAP and Table S1). A small subset, comprising seven isolates from clinical samples only, displayed MICs ≥ 4 $\mu\text{g/ml}$; such high MICs to daptomycin have not been

previously reported, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and The British Society for Antimicrobial Chemotherapy (BSAC) surveillance data. These MICs were repeated a second time and confirmed. This is concerning given that daptomycin is a current therapeutic choice for treating soft tissue infections caused by NAS (21). Our ARIBA analysis (Table S2) indicated only a single *S. epidermidis* isolate with an MIC of 1 µg/ml contained genes implicated in daptomycin resistance: *gshF* and *liaFRS*. The remaining 169 isolates with an MIC ≥1 µg/ml did not harbour any of these genes. Several mutations or genes are associated with daptomycin resistance in *S. aureus* (including *mprF*, and SNPs in *rpoC*) but none of these were identified in the NAS collection (6, 22). SNPs in *walk* have also been associated with daptomycin resistance in *S. aureus* (23); this gene was identified by protein BLAST as present across the NAS collection but with several differences at the protein level which raises doubts about whether the SNPs observed in *S. aureus* can be directly extrapolated to NAS. We therefore conclude that there is a potentially novel daptomycin resistance mechanism present in these strains, which we are evaluating further.

Resistance to vancomycin found in clinical samples

Vancomycin is a treatment option in prosthetic joint infection, and 94 % of isolates had an MIC below 4 µg/ml (Figure 1 VAN). However, of the 24 isolates with reduced susceptibility, 22 (92 %) came from clinical samples and only 2/24 were found in healthy volunteers. This is indicative of a wider trend, where isolates associated with clinical samples had significantly higher MICs ($p < 0.005$) than non-clinical isolates for cefoxitin, erythromycin, gentamicin, tetracycline, daptomycin and vancomycin (Figure S1). Given the importance of NAS in nosocomial infections, this is a worrying prospect both in terms of what is presenting in the

clinic and also the possibility of AMR gene transfer into organisms more capable of causing infection, including *S. aureus*. In addition, no known mechanisms of resistance were identified for vancomycin, rifampicin or teicoplanin (Figure S2 and Table S3).

Over half of the NAS collection displayed susceptibility to multiple antimicrobials

Out of the all the isolates tested, 49 % (187/394) had reduced susceptibility to three or more antimicrobials. Twenty-four isolates had reduced susceptibility to six antimicrobials, and three isolates had reduced susceptibility to seven antimicrobials; of these 23/24 and 3/3 were isolated from clinical samples (Table S1). The implications of these are difficult to treat infections and potentially a large reservoir of staphylococcal resistance genes within the patient under antimicrobial treatment.

Animal isolates have similar MIC distributions to human isolates

It is generally acknowledged that the presence of reduced susceptibility in microorganisms isolated from animals can impact upon public health if those organisms also cause infection in humans (24, 25). In our collection there were 40 NAS isolated from animals (including 7 NCTC strains), of which we obtained genome sequences from 29. Although in much fewer numbers than the human isolates in the collection, the animal isolates displayed very similar MIC distributions and harboured corresponding genetic mechanisms. This does not rule out the possibility that animals could be a reservoir of AMR for staphylococci.

Conclusion

Genome analysis of isolates displaying MICs to cefoxitin of ≥ 4 $\mu\text{g/ml}$ indicated that approximately half harboured the *mecA* element. The absence of *mecA* from the other half

suggests that other mechanisms are likely present. This was apparent across many of the antimicrobials tested as between 0 and 70 % of phenotypic resistance in clinical isolates could be attributed to known resistance mechanisms. The remaining 30-100 % suggests that there are potentially numerous unknown mechanisms underpinning NAS resistance, which warrant further investigation.

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Transparency declarations

None to declare.

References

1. Becker K, Heilmann C, Peters G. Coagulase-Negative Staphylococci. Clinical Microbiology Reviews. 2014;27(4):870-926.
2. NJR. 14th Annual Report 2017. In: National Joint Registry for England W, Northern Ireland and the Isle of Man, editor. 2017.
3. Lamagni T. Epidemiology and burden of prosthetic joint infections. J Antimicrob Chemother. 2014;69:i5-i10.
4. Aggarwal V, Bakhshi. H., Ecker, NU., Parvizi, J., Gehrke, T., Kendodd, D. Organism profile in periprosthetic joint infection: pathogens differ at two arthroplasty infection

referral centers in Europe and in the United States. The Journal of Knee Surgery 2014;27(399-406).

5. Morgenstern M, Erichsen, C., Hackl, S., Mily, J., Militz, M., Friederichs, J., Hungerer, S., Buhren, V., Moriarty, TF., Post, V., Richards, RG. and Kates, SL. Antibiotic resistance of commensal *Staphylococcus aureus* and coagulase-negative staphylococci in an international cohort of surgeons: a prospective point-prevalence study. . Morgenstern M, Erichsen C, Hackl S. 2016;11(2)::e0148437.
6. Sabat A, Tinelli M, Grundmann H, Akkerboom V, Monaco M, Del Grosso M, et al. Daptomycin Resistant *Staphylococcus aureus* Clinical Strain With Novel Non-synonymous Mutations in the *mprF* and *vraS* Genes: A New Insight Into Daptomycin Resistance. Front Microbiol. 2018;9:2705.
7. Swenson J, Brasso, WB., Ferraro, MJ., Hardy, DJ., Knapp, CC., Lonsway, D., McAllister, S., Reller, LB., Sader, HS., Shortridge, D., Skov, R., Weinstein MP., Zimmer, BL., and Patel, JB. Correlation of Cefoxitin MICs with the Presence of *mecA* in *Staphylococcus* spp. J Clin Microbiol 2009;47(6):1902-5.
8. Babraham Bioinformatics. FastQC: A quality control tool for high throughput sequence data [Available from: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.
9. Bolger A, Lohse, M., and Usadel, B. . Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
10. Institute JG. BBNorm: Kmer-based error-correction and normalization tool. [Available from: <https://sourceforge.net/projects/bbmap/>.
11. Jia B, Raphernua, AR., Alcock, B., Waglechner, N., Guo, P., Tsang, KK., Lago, BA., Dave, BM., Pereira S., Sharma, AN., Doshi, S., Courtot, M., Lo, R., Williams, LE., Frye, JG., Elsayegh, T., Sarder, D., Westman, EL., Pawlowski, AC., Johnson, TA., Brinkman, FS., Wright, GD. and McArthur, AG. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 2017;45(D1):D566-D73.
12. Hunt M, Mather, AE., Sánchez-Busó, L., Page, AJ., Parkhill, J., Keane, JA., Harrish, SR. ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. Microb Genom. 2017;3(10):e000131.
13. Seemann T. ABRicate: mass screening of contigs for antibiotic resistance genes. 2016.
14. EUCAST. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Version 2.0: The European Committee on Antimicrobial Susceptibility Testing; 2017 [updated March 2018. Available from: <http://www.eucast.org>.
15. Ferretti J, Gilmore, K., Courvalin, P. Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2"-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J Bacteriol. 1986;167:631-8.
16. Chow J. Aminoglycoside resistance in *enterococci*. Clin Infect Dis. 2000;31:586-9.
17. Perreten V, Kadlec, K., Schwarz, S., Grönlund Andersson, U., Finn, M., Greko, C., Moodley, A., Kania, SA., Frank, LA., Bemis, DA., Franco, A., Iurescia, M., Battisti, A., Duim, B., Wagenaar, JA., van Duijkeren, E., Weese, JS., Fitzgerald, JR., Rossano, A., Guardabassi, L. . Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study. J Antimicrob Chemother 2010;65:1145-54.

18. Schmitz F-J, Fluit, AC., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Heinz, HP., Jones, ME. The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. J Antimicrob Chemother. 1999;43:253-9.
19. Neuert S, Nair, S., Day, MR., Doumith, M., Ashton, PM., Mellor, KC., Jenkins, C., Hopkins, KL., Woodford, N2., de Pinna, E., Godbole, G., Dallman, TJ.,. Prediction of Phenotypic Antimicrobial Resistance Profiles From Whole Genome Sequences of Non-typhoidal Salmonella enterica. Front Microbiol. 2018;9:592.
20. Sapula SA, Brown MH. Antimicrobial Drug Efflux Pumps in Staphylococcus aureus. In: Li X-Z, Elkins CA, Zgurskaya HI, editors. Efflux-Mediated Antimicrobial Resistance in Bacteria: Mechanisms, Regulation and Clinical Implications. Cham: Springer International Publishing; 2016. p. 165-95.
21. White B, Seaton, RA. . Complicated skin and soft tissue infections: literature review of evidence for and experience with daptomycin. Infect Drug Res. 2011;4:115.
22. Friedman L, Alder, JD., Silverman, JA. . Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother 2006;50:2137-45.
23. Yang S, Mishra, NN., Rubio, A., Bayer, AS. Additional routes to Staphylococcus aureus daptomycin resistance as revealed by comparative genome sequencing, transcriptional profiling, and phenotypic studies. Antimicrobial Agents and Chemotherapy. 2013;57(11):5658-64.
24. Landers T, Cohen, B., Wittum, TE., Larson, EL. A review of antibiotic use in food animals: perspective, policy, and potential. . Public Health Rep. 2012;127:4-22.
25. Woolhouse M, Ward, M., van Bunnik, B. . Antimicrobial resistance in humans, livestock and the wider environment. Phil Trans R Soc B. 2015;370:20140083.

Figure legends

Figure 1. Antibiotic susceptibility of NAS collection

MIC distributions for 400 NAS isolates grown in the presence of antimicrobials. FOX cefoxitin, GEN gentamicin, TET tetracycline, ERY erythromycin, DAP daptomycin, VAN vancomycin, RIF rifampicin and TEC teicoplanin.

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

387 Per staphylococcal species, bars display the number of isolates found to have a ceftazidime
 388 MIC of ≥ 4 $\mu\text{g/ml}$ [red] or < 4 $\mu\text{g/ml}$ [peach] (4 isolates had no MIC data [blue], in relation to
 389 the presence [stripe] and absence [dots] of *mecA*. Sum of the MIC bar is the total number of
 390 isolates sequenced for that species.

391

392

393

Table 1

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

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

*Species designated by MALDI-TOF (Bruker)

Table 2 Genetic mechanisms of resistance identified with ARIBA and ABRicate using CARD database compared to the MIC data (of sequenced isolates only) using 90 % match ID (partial and interrupted sequences are not included, see table S2)

<i>Antimicrobial</i>	<i>Mechanism (Accession No.)</i>	<i>No. isolates above breakpoint MIC ($\geq 2 \mu\text{g/ml}$)</i>		<i>No. isolates below breakpoint</i>
TET	<i>tetK</i> (NC_013452)	48/148 (32.4%)		8/223 (3.6 %)
	<i>tetL</i> (M11036.0)	1/148 (0.7 %)		0/223
	<i>tetM</i> (AM180355)	1/148 (0.7 %)		0/223
	All	50/148 (33.8 %)		8/232 (3.6 %)
		<i>No. isolates above breakpoint MIC ($\geq 1 \mu\text{g/ml}$)</i>		
GEN	<i>aac(6')-Ie-aph(2'')-Ia</i> (NC_005024)	49/130 (37.7 %)		6/ 246 (2.4 %)
	<i>aph(3)IIIa</i> (CP004067)	13/ 130 (10 %)		7/ 246 (2.8 %)
	All*	59^{g1}/130 (22.3%)		12^{g2}/246 (4.9 %)
		<i>MIC $\geq 512 \mu\text{g/ml}$</i>	<i>MIC $\geq 2 - 256 \mu\text{g/ml}$</i>	
ERY	<i>msrA</i> (NC_022598.1)	6/ 66 (9 %)	75/135 (55.5 %)	9/171(5.2 %)
	<i>ermC</i> (M12730)	42/ 66 (63.6 %)	13/135 (9.6 %)	9/171 (5.2 %)
	<i>ermA</i> (NC_009632)	6/ 66 (9%)	1/135 (0.7 %)	0/171
	<i>emeA</i> (AB091338)	1/ 66 (1.5 %)	0/135	0/171
	All*	52^{e1}/66 (78.7 %)	88^{e2}/135 (65.2 %)	18/171 (10.5 %)

*Some isolates harboured multiple resistance genes. TET tetracycline, GEN gentamicin, ERY erythromycin; g1: 3 - isolates contained multiple resistance genes; g2: 1 isolate contained multiple resistance genes; e1: 3 isolates contained multiple resistance genes; e2; 1 isolate contained multiple resistance genes

Supplementary data

410 Table S1. MIC characterisation of the NAS collection

411 MIC data for all isolates tested; all MIC are shown in µg/ml; FOX cefoxitin, GEN gentamicin,
412 TET tetracycline, ERY erythromycin, DAP daptomycin, VAN vancomycin, RIF rifampicin and
413 TEC teicoplanin.

414

415 Table S2. ARIBA characterisation of all sequenced NAS isolates, grouped by relevant
416 antimicrobial.

417

418 Table S3. Summary of ARIBA/ABRicate match data for all sequenced isolates.

419

420 Table S4. Comparison of gene presence (using ARIBA) and protein BLAST presence. Data
421 includes all genes identified in the CARD database.

422

423 Figure S1. MICs in clinical and non-clinical isolates

424 Box and whisker plot of MIC distribution per antimicrobial for clinical (orange) and non-
425 clinical (yellow) isolates. Thick black bar indicates the median MIC which resided within the
426 Interquartile Range. The extreme lines are represented as dotted lines and indicate the data
427 outside the upper (75%) and lower (25%) quartiles, open circles represent potential outliers.

428 Levels of significance from Mann-Whitney U test denoted by **** (p<0.0001), ***
429 (p<0.005), ** (p=0.001) and * (p=0.01).

430

431 Figure S2. Mechanisms of resistance

432 Percentage of known genetic mechanisms identified in sequenced NAS isolates with
433 reduced susceptibility. Number of isolates with reduced susceptibility per antimicrobial

434 given in parentheses. Known mechanisms found per antimicrobial are shown in italics. FOX
435 cefoxitin, GEN gentamicin, TET tetracycline, ERY erythromycin, DAP daptomycin, VAN
436 vancomycin, RIF rifampicin and TEC teicoplanin.
437