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An observational cross-sectional study of nasal staphylococcal species of medical students of diverse geographical origin, prior to healthcare exposure. Prevalence of SCC*mec*, *fusC, fusB* and the arginine catabolite mobile element (ACME) in the absence of selective antibiotic pressure.

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Keywords; *Staphylococcus aureus,* Coagulase-negative staphylococci, Nasal colonization, Healthy human nares, Antimicrobial resistance, MRSA.

Running title: Antimicrobial resistance and virulence genes among staphylococcal carriage isolates.

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Abstract

Objective: The aim of this study was to investigate co-located nasal *S. aureus* and CoNS (mainly *S. epidermidis*) recovered from healthy medical students in their pre-clinical year, prior to exposure to the healthcare environment, for the carriage of genes and genetic elements common to both species and that may contribute to *S. aureus* and MRSA evolution.

Design: Prospective observational cross-sectional study. Carriage of antimicrobial resistance and virulence-associated genes in the absence of significant antibiotic selective pressure was investigated among healthy medical students from geographically diverse origins who were nasally co-colonised with *S. aureus* and CoNS. Clonal lineages of *S. aureus* isolates were determined.

Setting/Participants: Dublin-based international undergraduate medical students

Results: Nasal *S. aureus* carriage was identified in 137/444 (30.8%) students of whom nine (2%) carried MRSA (ST59-MRSA-IV (6/9), CC1-MRSA-V-SCC*fus* (3/9)). The genes *mecA*, *fusB*, *ileS2, qacA*/*qacC* and ACME-*arc* were detected among colonizing nasal staphylococci and had a significantly greater association with CoNS than *S. aureus.* The rate of co-carriage of any of these genes in *S. aureus*/CoNS pairs recovered from the same individual was <1 %.

Conclusions The relatively high prevalence of these genes among CoNS of the healthy human flora in the absence of significant antibiotic selective pressure is of interest. Further research is required to determine what factors are involved and whether these are modifiable to help prevent the emergence and spread of antibiotic resistance amongst staphylococci.

Strengths and limitations of this study:

- Evaluation of resistance gene carriage among Staphylococci in healthy medical students in preclinical years.
- Evidence of antibiotic resistance among Staphylococci in the absence of selective pressure.
- Study was carried out in a single centre.
- CoNS was investigated only in students co-colonised with *S. aureus*.
- The study design did not facilitate follow-up of this cohort during clinical training.

Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* are significant colonisers of healthy human skin and nares and are among the leading causes of healthcare-associated infection (HAI). Morbidity, mortality and the financial burden associated with methicillin-resistant *S. aureus* (MRSA) infections are well documented. Furthermore, coagulase-negative staphylococci (CoNS) including *S. epidermidis* are reported reservoirs of antimicrobial-resistance genes and their associated mobile genetic elements, most notably the staphylococcal cassette chromosome (SCC) harbouring the *mec* gene (SCC*mec*) [1].

Twelve SCC*mec* types and numerous subtypes have been described among MRSA isolates to date. The more prevalent and diverse range of SCCs and SCC*mec* among CoNS further supports CoNS as a reservoir for antimicrobial resistance genes [1]. The identification of SCC, SCC*mec* and SCC-associated elements with other antimicrobial and virulence genes and their epidemiological relationships among clinical staphylococci has advanced our understanding of the role of CoNS in the evolution of MRSA [2]. For example, the fusidic acid resistance gene *fusC* is associated with SCC*mec*IV-SCC_{476} and other SCC-like elements have been identified in *S. aureus*, MRSA and CoNS and may contribute to MRSA emergence in countries

with significant fusidic acid usage. [3-5] Furthermore, the SCC-like arginine catabolic mobile element (ACME) which enhances acid tolerance, is abundant among clinical CoNS isolates, in particular *S. epidermidis* and *S. haemolyticus* [6]. Among *S. aureus,* ACME has mainly been detected among isolates of the community-associated (CA) USA300 clone [7]. CoNS are also a putative reservoir of the high level mupirocin resistance encoding gene *ileS2*, which is also increasing among *S. aureus*/MRSA in healthcare and community environments related to horizontal gene transfer or expansion of specific clones [8 9].

Increasingly, MRSA clones previously associated with the community, such as CC1 are spreading to healthcare settings making the differentiation between healthcare-associated (HA) MRSA and CA-MRSA unclear [10]. Therefore, detailed investigation of the genetic and phenotypic traits of colonizing staphylococcal species in community settings are important to identify those with features that may contribute to their evolution into potentially successful and formidable healthcare-associated clones. The aim of this study was to investigate co-located nasal *S. aureus* and CoNS (mainly *S. epidermidis*) recovered from healthy medical students in their pre-clinical year, prior to exposure to the healthcare environment, for the carriage of genes and genetic elements common to both species and that may contribute to *S. aureus* and MRSA evolution.

Methods

Study setting, participants and sample collection

The study was conducted at the Royal College of Surgeons in Ireland (RCSI) from December 2014 – January 2016. Nasal swabs (eSwab Copan®), Italy) were collected anonymously from undergraduate medical students. Eligible students were those attending the RCSI medical centre to submit a swab for mandatory MRSA screening in the week before they began their clinical attachments. In total 444/450 eligible medical

students (250 (56.3%) male, 194 (43.7%) female) participated in this study. All participants reported no previous hospital contact in the six weeks prior to recruitment. The student volunteers were from the second year of the undergraduate medical programme and as such, all participants were domiciled in Ireland for a minimum of two years prior to recruitment. Data was collected anonymously from each participant, including age range, region of origin and previous healthcare contact. Ethical approval (approval number REC949) was obtained from the Institute's Ethics Committee and informed consent was obtained from each participant.

Sample preparation

Swabs were processed to recover *S. aureus* (including MRSA) and CoNS using a modification of a published method[11]. Swabs were enriched in brain heart infusion (BHI) supplemented with 6% (w/v) NaCl for 24 h at 37°C followed by further enrichment in mannitol salt broth for 24 h at 37°C. The enriched culture was diluted 1/1000 and 100 µl was spread onto SaSelect agar (Bio-Rad®, Hercules, CA, USA). Plates that yielded pink/orange colonies (presumptive *S. aureus*) were inspected for growth of colonies of relevent CoNS (e.g. light pink colonies of various sizes, presumptive *S. epidermidis*; white/yellow colonies, *S. haemolyticus*, *S. hominis*, *S. capitis*, *S. warneri*, *S. caprae*, *S. lugdunensis*). Presumptive CoNS species and *S. aureus* were sub-cultured from these plates onto Columbia blood agar (CBA) and identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF) using a MALDI Biotyper (Microflex LT, Brüker). Matched isolates (where *S. aureus* and a CoNS species were recovered from the same swab) were cryopreserved and stored at -20°C (Protect™ bacterial preserver beads (Technical Service Consultants, UK).

Characterisation of *S. aureus* and CoNS isolates

Genomic DNA from *S. aureus* and CoNS isolates was extracted using enzymatic lysis using the buffers and solutions provided with the *S. aureus* Genotyping Kit 2.0 (Alere

Technologies GmbH, Jena, Germany) and a DNeasy® Blood and Tissue kit (Qiagen, Crawley, UK). Genetic characterisation of isolates was undertaken by DNA microarray profiling using the *S. aureus* Genotyping Kit 2.0 as described previously [12 13]. The kit detects 333 gene targets including staphylococcal antimicrobial-resistance, virulence, SCC*mec* and ACME-*arc* genes and assigns *S. aureus* isolates to multilocus sequence type (ST) or clonal complexes (CC)s. MRSA phenotype was confirmed in *S. aureus* and CoNS isolates positive for *mecA* by growth of pink (*S. aureus*) or colorless/white (CoNS) colonies on MRSASelect agar (Bio-Rad®, Hercules, CA, USA). When required, confirmation of carriage of *fusC, fusB* and *tst1* was confirmed by PCR using the primers and conditions described by O'Neill et. al. [14] and Chen et. al. [15]

Fusidic acid susceptibility testing

Fusidic acid MICs were determined by ETEST® (bioMérieux, Marcy-l'Etoile, France) according to manufacturer's instructions. Thirty *S. aureus* and CoNS isolates harbouring fusidic acid resistance genes (*fus*C or *fus*B) were sub-cultured twice before testing. Results were interpreted according to EUCAST (http://www.eucast.org, assessed May 2015) susceptibility criteria.

Statistical analyses

Fisher's exact test was used to analyze categorical variables (prevalence of genes) using GraphPad QuickCalcs on-line software. The significance of differences between groups was expressed as two-tailed p-values, p values of ≤ 0.05 were considered statistically significant.

Results

Nasal carriage of staphylococcal species and regional distribution

Thirty-one percent (137/444) of students were positive for nasal carriage of *S. aureus* of whom 6.5 % (9/137) were MRSA. Eighty-seven percent (386/444) of students were positive for nasal carriage of CoNS (*S. epidermidis* (82% 364/444), *S. haemolyticus* (3% 14/444) or *S. saprophyticus* (2% 8/444). Methicillin-resistant (MR)-CoNS were investigated in the *S. aureus*-positive cohort only of which 13.1 % (18/137) were MR-CoNS. One student exhibited co-carriage of MRSA and MR-CoNS. The geographical region of origin of students harbouring *S. aureus* and CoNS is shown in Figure 1. The Middle East, Europe and North America accounted for 68.6% of *S. aureus* carriers. For regions represented by ≥ 12 participants, the rate of nasal carriage of *S. aureus* varied geographically between 17 % (South East Asia) and 44 % (Africa).

Clonal lineages among *S. aureus* isolates

The ST or CC distribution among 137 *S. aureus* isolates is shown in Figure 2. Isolates belonged to a variety of CCs with 46/137 (33.5%) assigned to internationally disseminated CC5, CC8, CC22, CC30, CC45. A further 24/137 (17.5%) isolates belonged to CC1, CC59, CC88 or CC398.

SCC*mec* types and fusidic acid resistance among *S. aureus* and CoNS

Of the 333 staphylococcal genes detected by the microarray, the two most prevalent antibiotic resistance genes among nasal staphylococci were those encoding resistance to β-lactams and fusidic acid. The most common SCC*mec* type among nasal MRSA (n=9) and MR-CoNS (n=18) was SCC*mec* type IV. The nine MRSA isolates belonged to ST59-MRSA-IV (6/9) and CC1-MRSA-V-SCC*fus* (3/9). Among 18 MR-CoNS identified (17 *S. epidermidis* and 1 *S. saprophyticus*), half harboured SCC*mec* type IV (8 *S. epidermidis* and the single *S. saprophyticus*). SCC*mec* types II, V and VII were identified in three, five and one of the remaining *S. epidermidis* isolates, respectively.

Isolates from the one individual who exhibited nasal co-carriage of MRSA and MR-CoNS (*S. epidermidis*) both harboured SCC*mec* type IV (Table 1).

In addition to the three CC1-MRSA-V isolates that carried SCC*fus*, the fusidic acid resistance genes *fusC* and *fusB* were identified in 28/128 (21.8%) and 2/128 (1.5%) of methicillin-susceptible *S. aureus* (MSSA) isolates, respectively. Ten of the 28 *fusC*-positive MSSA isolates belonged to CC1-MSSA-SCC*fus* and 18 were CC88-MSSA. All 10 CC1-MSSA-SCC*fus* isolates harboured a combination of SCC*fus* with the *ccr* genes *ccr*A1 *ccr*B-1. The two *fus*B positive isolates belonged to CC5-MSSA and CC8-MSSA (Table 1). Among MR-CoNS, 27.7 % (5/18) *S. epidermidis* isolates carried *fus*C (two of them also carried *ccr* genes *ccr*A1 *ccr*B-1) and 50 % *fusB* (9/18, eight *S. epidermidis* and the one *S. saprophyticus*). Among methicillin susceptible CoNS isolates, the *fus*C and *fus*B genes were identified in 20/119 (16.8%, 18 *S. epidermidis* and two *S. saprophyticus*) and 18/119 (15.1%, all *S. epidermidis*), respectively. One participant had nasal co-carriage of *fusC*-positive *S. aureus* (CC88-MSSA) and CoNS (*S. epidermidis*).

All SCC*mec* positive staphylococci were confirmed to have an MRSA/MR-CoNS phenotype. However, there was poor correlation between *fusC/fusB* carriage and phenotypic fusidic acid resistance. Fusidic acid MICs for all *fusC* or *fusB*-positive *S. aureus* and CoNS isolates are shown in Table 2. Phenotypic fusidic acid resistance was confirmed (based on EUCAST breakpoints, MICs ≥1 μg/ml) in 23/32 (71.8%) *S. aureus* and 20/38 (52.6%) CoNS nasal isolates harbouring either *fus*C or *fus*B (DNA microarray result confirmed by PCR). Eight nasal isolates (three *S. aureus*, five *S. epidermidis*) positive for *fusB* exhibited high level fusidic acid resistance (MIC≥ 32 μg/ml). Fusidic acid resistance was inducible in a further three *S. aureus* and seven *S. epidermidis* isolates following incubation with 0.01 μg/ml fusidic acid BHI agar.

Other notable antimicrobial resistance genes among nasal *S. aureus* and CoNS

Apart from SCC*mec* element and *fus* genes, other antimicrobial genes detected among staphylococcal nasal flora were identified by DNA microarray. Tetracycline resistance genes, *tet(*K) or *tet(*M), were detected in 13/137 (9.5 %) of *S. aureus* isolates and 6/137 (4.3%) of the CoNS isolates. The quaternary ammonium compound resistance genes (*qac*A/*qac*C), encoding antiseptic resistance, were significantly more prevalent among CoNS isolates compared to *S. aureus* isolates (29/137 (21.2%) *Vs* 2/137 (1.4%), p<0.0001). Significantly more CoNS than *S. aureus* isolates carried *ileS2* encoding high-level mupirocin resistance (11/137 (8%) *vs* 1/137 (0.72%), p<0.01). However, none of these genes were common to *S. aureus*/CoNS pairs recovered from the same individual. The β-lactamase genes were abundant among *S. aureus* and CoNS; *bla*Z was present in 101/137 (73.72%) *S. aureus* isolates and 92/137 (67.1%) CoNS isolate and in 74/137 (54%) of individuals, these genes were common to *S. aureus*/CoNS pairs from the same nares. A summary of the antibiotic resistance genes found among *S. aureus* and CoNS is shown in Table 1. The staphylococcal isolates were negative for all other antibiotic resistance genes detected by the microarray.

Virulence genes among nasal *S. aureus* and CoNS

A single isolate, CC30-MSSA, was positive for the Panton-Valentine leucocidin genes (*lukF/S-PV*). Among nasal staphylococci, ACME-*arc* was significantly associated with CoNS compared to *S. aureus* (44/137 (32.1%) Vs 1/137 (0.7%)), p<0.0001. The toxic shock syndrome toxin gene *tst1* was identified in 33/137 (24.1%) nasal *S. aureus* isolates. Unusually, DNA microarray identified *tst1* in two *S. epidermidis* isolates and this was confirmed by PCR. ACME-*arc* was common to *S. aureus*/CoNS recovered from the nares in one individual only. One hundred and two (74.4%) *S. aureus* isolates encoded one or more enterotoxin genes. The enterotoxin gene cluster (egc), containing *seg, sei, sem, sen, seo, seu*) was the most prevalent (48/102, 47%) followed by *seq/k* (13/102, 12.7 %) and *sec/l* (7/102, 6.8 %). Staphylococcal isolates were negative for all other toxin genes detected by the microarray.

Discussion

Studies of staphylococcal carriage and epidemiology among the healthy population in the absence of significant antibiotic pressure are important in identifying the potential for pathogenic evolution. To our knowledge, this is the first study to co-investigate CoNS and *S. aureus* when recovered together from the nares of healthy pre-clinical medical students. Our study revealed that, apart from the *bla* genes, which are abundant among staphylococci, the rates of co-carriage of antibiotic resistance genes in paired *S. aureus*/CoNS from the same individual were low in the community setting at <1%. Rates of simultaneous carriage of antimicrobial resistance among nasal staphylococci are likely to be higher under selective antibiotic pressure but few studies have investigated this among patients. One small study of hospitalized patients with nasal carriage of *S. aureus* and CoNS reported a rate of 12.5 % patients carrying MRSA and MR-CoNS [16]. However, the authors reported only two cases where simultaneous carriage of MR-CoNS and MRSA was detected and the strains involved carried different SCC*mec* types. Despite negligible detection of co-species nasal carriage of these genes in medical students prior to healthcare exposure, based on antimicrobial resistance gene carriage by CoNS from this cohort, there is significant potential for mobilisation of genes to *S. aureus* that may enhance its pathogenic potential in the healthcare setting.

DNA microarray analyses revealed carriage of SCC*mec, fusC, fusB, ileS2, qacA/qacC* and ACME-*arc* among colonising nasal staphylococci in individuals with no previous healthcare exposure with greater prevalence among CoNS than *S. aureus*. This pattern among pre-clinical medical students, supports CoNS as a reservoir with potential to subsequently accelerate antimicrobial resistance and pathogenicity among colonizing *S. aureus* in clinical environments under antibiotic selective pressure [17-19].

Despite considerable geographical distribution of the participants in this study, a *S. aureus* nasal carriage rate in the community of 30.8% was recorded. In this study, CC30, CC88 and CC8 were the most prevalent clones identified among nasal *S. aureus*. CC30 is among the internationally disseminated clones in which SCC*mec* has been acquired and is a successful colonising strain, reported among HA and CA-MRSA. CC88 is frequently isolated in Australia but in our study the geographical background of isolates was mixed (including Middle East, Europe, South East Asia and Central America). CC8 is associated with MRSA infection and is globally disseminated [20]. Two CC/ST types were detected among MRSA recovered from healthy medical students in this study, ST59-MRSA-IV and CC1-MRSA-V+SCC*fus*C. ST59 (WA-MRSA-73) is a sporadic Australian strain and apart from PVL-negativity, is indistinguishable from USA1000 [21] In this study the geographical background of these isolates was wide (Middle East, North America and South East Asia).

The identification of a significant reservoir of antibiotic resistance among medical students prior to healthcare exposure in subsequent clinical years, highlights the need for effective infection prevention and control policies in relation to hand hygiene and surveillance. In the absence of antibiotic selective pressure, the colonising MRSA rate appears relatively stable and in this study was 2 % (9/444), similar to rates reported elsewhere [22]. However, a previous study among medical interns in China reported a nasal MRSA rate of 9.4% likely reflecting exposure to the healthcare environment [23]. Prevalence rates of MR-CoNS in recent community-based surveys are variable but rates of 16.5% [18] and 17.2% [24] are reported in similar cohorts to this study where, of those colonised with *S. aureus*, 13.1% carried MR-CoNS. SCC*mec* type IV, the smallest of the SCC*mec* elements, was the most prevalent type among MRSA and MR-CoNS here (66.6% and 50%). SCC*mec* IV has been detected in approximately 40% of methicillin-resistant *S. epidermidis* identified in humans [25]. However, in this study SCC*mec* type V was also represented among

MRSA and MR-CoNS. While only one individual was colonised with MRSA and MR-CoNS in this study (both SCC*mec* type IV), the preponderance of this SCC*mec* element in nasal MRSA and MR-CoNS suggests the potential for *mec*A gene transfer among these species even in the absence of selective pressure. The small size of this element, which has a low fitness cost, may enhance its dissemination potential. [26]

Fusidic acid resistance among *S. aureus* from healthy carriers in nine European countries in 2014 was reported to be <10 % [27]. However, we found a prevalence of 21.9% of *fusC/fusB* genes among healthy carriers. Fusidic acid resistance appears to correlate with increased use of this agent. For example, in New Zealand, where it is used as a first-line empiric agent for topical treatment of impetigo, prevalence rates of resistance in community *S. aureus* isolates increased from 17% in 1999 to 29% in 2013 [28]. In Europe, fusidic acid is combined with β-lactams for the treatment of staphylococcal bacteraemia, endocarditis, and osteomyelitis [29] and is used widely in the community for SSTIs. A 2010 study of fusidic acid resistance among *S. aureus* clinical isolates showed Greece and Ireland to have the highest rates (52.5 and 19.9%) [30]. SCC*fus* has been identified in the CC1 background and more recently, in other lineages such as ST239 and ST779 [31-33]. As highlighted here in the absence of significant antibiotic pressure in the community, it appears that this element is associated with MRSA and MSSA in the CC1 background. This genetic platform, particularly when associated with SCC*mec*, may enable the transfer of multi-drug resistance on a single mobile element. The use of fusidic acid is un-regulated in some countries and hence it may be used inappropriately in a community setting (for example in short or discontinuous doses). Inappropriate use of fusidic acid may therefore favour co-selection of methicillin-resistance among *S. aureus*. In addition, in this study, 14/18 (77%) of MR-CoNS were positive for *fusC* or *fusB*. This association of resistances among the resident flora may provide further opportunity for dissemination of MRSA driven by fusidic acid selective pressure. Interestingly a positive correlation

between carriage of *fusC/fusB* and phenotypic resistance was observed in only 71.9 % and 53.6 % of *S. aureus* and CoNS respectively. However, induction of gene expression with fusidic acid pre-incubation gave better correlation (82.2% and 76.3 % correlation).

There were limitations to this study, which included; a single centred, relatively small study. CoNS was investigated only in those co-colonised with *S. aureus*. CCs and STs were determined only among *S. aureus* as the high rate of genetic recombination among CoNS makes strain typing unreliable. Although the microarray system used is reported effective for staphylococcal species other than *S. aureus* [34], some gene targets may be heterologous among staphylococci leading to false negatives. The study design did not facilitate follow-up of this cohort during clinical training which may have revealed further changes in gene carriage among colonising staphylococci. However, the multi-national origin of the student body in our institution facilitated analysis of a relatively broad geographic cohort in a single study and emphasises the role that importation plays in *S. aureus* epidemiology. Unlike other studies of staphylococci in the healthy human nares, pairs of staphylococcal species originating from the same individual were investigated here for their resistance and virulence traits. These data support a low rate of transfer of antibiotic resistance between colonising staphylococcal species in the absence of healthcare contact. However, it is concerning that similar SCC*mec* and SCC*fus*C types, in addition to *ileS2, qacA/qacB* and ACME are carried among CoNS and *S. aureus* in healthy individuals who will have subsequent roles in healthcare provision. Given the increasing emergence of HA-MRSA with features of community strains, further mobilisation of these elements under selective antibiotic pressure may enhance the transmission and success of *S. aureus* in the healthcare environment.

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Competing Interests Statement

HH has received funding from Pfizer and Astellas outside the relevance of the submitted work. All other authors report no competing interests.

Contributorship statement

PEB and DFH recruited students to the study, PEB conducted the laboratory work and drafted the manuscript. DFH and HH conceived of the study and contributed to study design. AS, PK and DC provided critical data interpretation and revised the drafted work. All authors contributed to the final approved draft.

Data Sharing Statement All data for these analyses are included in the manuscript. No additional data are available.

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Figure Legends

Figure 1. The geographical origin of 444 medical students from whom a nasal swab was collected (dark grey bars) and the proportion of participants with nasal co-carriage of *S. aureus* and CoNS (light grey bars).

Figure 2. Genotypic diversity of 137 *S. aureus* nasal isolates using DNA microarray analysis, including 128 MSSA (grey bars) and 9 MRSA (white bars). Bold lettering indicates internationally disseminated clones into which SCC*mec* can integrate. CC=clonal complex

377 Table 1. Resistance/virulence genes detected among *S. aureus* and CoNS nasal isolates.

Detected Gene(s)	**Phenotypic resistance/trait**	**No. isolates positive n (%)**			**No. *S. aureus*/CoNS pairs positive (n)**
		S. aureus **n=137**	**CoNS n=137**	***P* value**	
Antibiotic resistance gene					
blaZ	β-lactam	101 (73.7)	92 (67.1)	0.289	74
fusB	Fusidic acid	2 (1.5)	18 (13.3)	0.0002*	0
fusC[a]	Fusidic acid	30 (21.9)	20 (14.5)	0.159	2
mecA	Methicillin	9 (6.5)	18 (13.1)	0.103	1
ileS2	Mupirocin	1 (0.7)	11 (8.0)	0.005*	1
*qac*A and *qac*C	Quartenary ammonium salts	3 (2.2)	29 (21.2)	<0.0001*	0
tet(K) and tet(M)	Tetracycline	13 (9.5)	6 (4.4)	0.152	0
erm(C)	Macrolide/lincosamide	6 (4.3)	5 (3.6)	1.000	0
msr(A)	Macrolide	2 (1.45)	15 (10.9)	0.002*	1
mph(C)	Macrolide	0	15 (10.9)	<0.0001*	0
dfrS1	Trimethoprim	0	19 (13.8)	<0.0001*	0

vga	Streptogramin A	1 (0.7)	6 (4.3)	0.120	0
Virulence					
ACME-*arc*	pH tolerance	1 (0.7)	44 (32.1)	<0.0001*	1
tst1[b]	Toxic shock toxin	33 (24.1)	2 (1.5)	<0.0001*	0

[a]associated with SCC element, (*ccr*A1 and *ccr*B-) in 13/137 *S. aureus*. [b]*tst1* confirmed by PCR. ACME = Arginine Catabolite Mobile Element. * indicates a statistically significant result by Fisher's exact test.

Table 2. Fusidic acid MICs for *S. aureus* and CoNS

MIC Interpretation[a]	MIC ≤ 1 µg/ml S n (%)	MIC ≥ 1 µg/ml R n(%)	MIC ≥ 32 µg/ml HR n (%)
***S. aureus* (n = 32)**	9 (28.1)	20 (62.5)	3 (9.3)
CoNS (n = 38)	18 (47.4)	15 (39.5)	5 (13.2)

[a]Interpretation based on The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1, 2017. http://www.eucast.org, S = Susceptible, R = Resistant, HR = high level resistant

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Figure 1

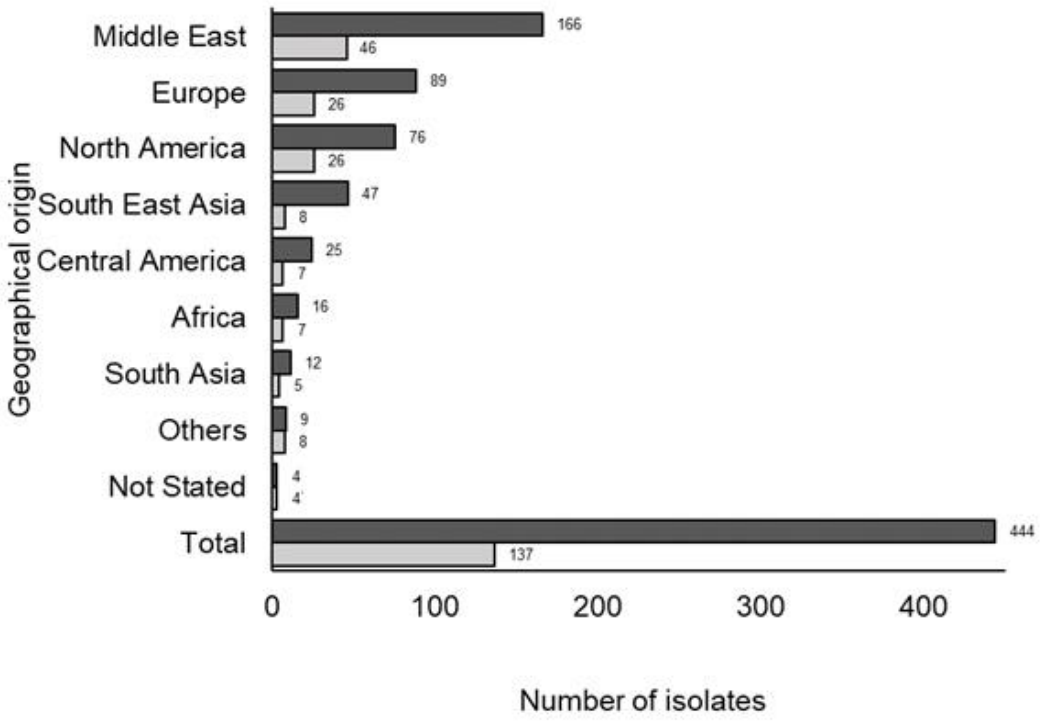

Figure 1. **Geographical origin of medical students recruited.** The geographical areas of origin of 444 medical students recruited to the study are shown (dark grey bars). Of those recruited, 137 were confirmed nasal S. aureus positive. The proportion of recruited students from each geographical origin with nasal S. aureus carriage are also shown (light grey bars).

Figure 2

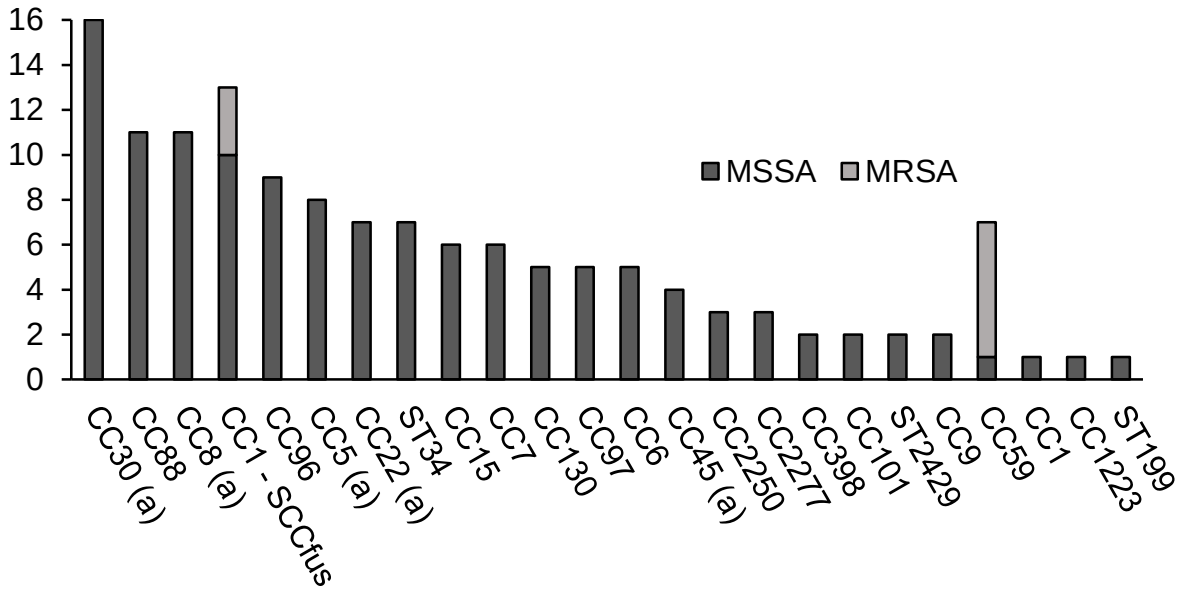

Figure 1. Genotypic diversity of 137 S. aureus nasal isolates using DNA microarray analysis, including 128 MSSA (grey bars) and 9 MRSA (white bars). Bold lettering indicates internationally disseminated clones into which SCCmec can integrate. CC=clonal complex.

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An observational cross-sectional study of nasal staphylococcal species of medical students of diverse geographical origin, prior to healthcare exposure. Prevalence of SCC*mec*, *fusC*, *fusB* and the arginine catabolite mobile element (ACME) in the absence of selective antibiotic pressure.

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An observational cross-sectional study of nasal staphylococcal species of medical students of diverse geographical origin, prior to healthcare exposure. Prevalence of SCC*mec*, *fusC*, *fusB* and the arginine catabolite mobile element (ACME) in the absence of selective antibiotic pressure.

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Running title: Antimicrobial resistance and virulence genes among staphylococcal carriage isolates.

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Abstract

Objective: The aim of this study was to investigate co-located nasal *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) (mainly *Staphylococcus epidermidis*), recovered from healthy medical students in their pre-clinical year, prior to exposure to the healthcare environment, for the carriage of genes and genetic elements common to both species and that may contribute to *S. aureus* and methicillin-resistant *S. aureus* (MRSA) evolution.

Design: Prospective observational cross-sectional study. Carriage of antimicrobial resistance and virulence-associated genes in the absence of significant antibiotic selective pressure was investigated among healthy medical students from geographically diverse origins who were nasally co-colonised with *S. aureus* and CoNS. Clonal lineages of *S. aureus* isolates were determined.

Setting/Participants: Dublin-based international undergraduate medical students

Results: Nasal *S. aureus* carriage was identified in 137/444 (30.8%) students of whom nine (6.6%) carried MRSA (ST59-MRSA-IV (6/9), CC1-MRSA-V-SCC*fus* (3/9)). The genes *mecA*, *fusB*, *ileS2, qacA*/*qacC* and the arginine catabolite mobile element (ACME)-*arc* were detected among colonizing nasal staphylococci and had a significantly greater association with CoNS than *S. aureus.* The rate of co-carriage of any of these genes in *S. aureus*/CoNS pairs recovered from the same individual was <1 %.

Conclusions The relatively high prevalence of these genes among CoNS of the healthy human flora in the absence of significant antibiotic selective pressure is of interest. Further research is required to determine what factors are involved and whether these are modifiable to help prevent the emergence and spread of antibiotic resistance amongst staphylococci.

Strengths and limitations of this study:

- Global evaluation of antibiotic resistance gene carriage among Staphylococci among healthy medical students in preclinical years through DNA microarray analyses.
- Pairs of staphylococcal species were isolated from the same colonisation site (nares) of multiple participants to allow investigation of shared antibiotic resistance and virulence in the same human niche in a community setting.
- A single centre study design.
- CoNS was investigated only in students co-colonised with *S. aureus*.
- The study design did not facilitate follow-up of this cohort during clinical training.

Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* are significant colonisers of healthy human skin and nares and are among the leading causes of healthcare-associated infection (HAI). Morbidity, mortality and the financial burden associated with methicillin-resistant *S. aureus* (MRSA) infections are well documented. Furthermore, coagulase-negative staphylococci (CoNS) including *S. epidermidis* are reported reservoirs of antimicrobial-resistance genes and their associated mobile genetic elements, most notably the staphylococcal cassette chromosome (SCC) harbouring the *mec* gene (SCC*mec*) [1].

Twelve SCC*mec* types and numerous subtypes have been described among MRSA isolates to date. The more prevalent and diverse range of SCCs and SCC*mec* among CoNS further supports CoNS as a reservoir for antimicrobial resistance genes [1]. The identification of SCC, SCC*mec* and SCC-associated elements with other antimicrobial and virulence genes and their epidemiological relationships among clinical staphylococci has advanced our understanding of the role of CoNS in the

evolution of MRSA [2]. For example, the fusidic acid resistance gene *fusC* is associated with SCC*mec* IV-SCC_{476} and other SCC-like elements have been identified in *S. aureus*, MRSA and CoNS and may contribute to MRSA emergence in countries with significant fusidic acid usage. [3-5] Furthermore, the SCC-like arginine catabolic mobile element (ACME) which enhances acid tolerance, is abundant among clinical CoNS isolates, in particular *S. epidermidis* and *S. haemolyticus* [6]. Among *S. aureus,* ACME has mainly been detected among isolates of the community-associated (CA) USA300 clone [7]. CoNS are also a putative reservoir of the high level mupirocin resistance encoding gene *ileS2*, which is also increasing among *S. aureus*/MRSA in healthcare and community environments related to horizontal gene transfer or expansion of specific clones [8 9].

Increasingly, MRSA clones previously associated with the community, such as clonal complex (CC) 1 are spreading to healthcare settings making the differentiation between healthcare-associated (HA) MRSA and CA-MRSA unclear [10]. Therefore, detailed investigation of the genetic and phenotypic traits of colonizing staphylococcal species in community settings are important to identify those with features that may contribute to their evolution into potentially successful and formidable healthcare-associated clones. The aim of this study was to investigate co-located nasal *S. aureus* and CoNS (mainly *S. epidermidis*) recovered from healthy medical students in their pre-clinical year, prior to exposure to the healthcare environment, for the carriage of genes and genetic elements common to both species and that may contribute to *S. aureus* and MRSA evolution.

Methods

Study setting, participants and sample collection

This observational cross-sectional study was conducted at the Royal College of Surgeons in Ireland (RCSI) from December 2014 – January 2016. Nasal swabs

(eSwab Copan®), Italy) were collected anonymously from undergraduate medical students. Eligible students were those attending the RCSI medical centre to submit a swab for mandatory MRSA screening in the week before they began their clinical attachments. In total 444/450 eligible medical students (250 (56.3%) male, 194 (43.7%) female) participated in this study. All participants reported no previous hospital contact in the six weeks prior to recruitment. The student volunteers were from the second year of the undergraduate medical programme and as such, all participants were domiciled in Ireland for a minimum of two years prior to recruitment. Data was collected anonymously from each participant, including age range, region of origin and previous healthcare contact. Ethical approval (approval number REC949) was obtained from the Institute's Ethics Committee and informed consent was obtained from each participant.

Sample preparation

Swabs were processed to recover *S. aureus* (including MRSA) and pathogenic CoNS species using a modification of a published method[11]. Swabs were enriched in brain heart infusion (BHI) supplemented with 6% (w/v) NaCl for 24 h at 37°C followed by further enrichment in mannitol salt broth for 24 h at 37°C. The enriched culture was diluted 1/1000 and 100 µl was spread onto SaSelect agar (Bio-Rad®, Hercules, CA, USA). Plates that yielded pink/orange colonies (presumptive *S. aureus*) were inspected for growth of colonies of relevant CoNS based on colony colour (e.g. light pink colonies of various sizes, presumptive *S. epidermidis*; white/yellow colonies, *S. haemolyticus*, *S. hominis*, *S. capitis*, *S. warneri*, *S. caprae*, *S. lugdunensis*). Presumptive CoNS species and *S. aureus* were sub-cultured from these plates onto Columbia blood agar (CBA) and identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF) using a MALDI Biotyper (Microflex LT, Bruker). Matched isolates (where *S. aureus* and a CoNS species were recovered from the same swab) were cryopreserved and stored at -20°C (Protect™ bacterial preserver beads (Technical Service Consultants, UK).

Characterisation of *S. aureus* and CoNS isolates

Genomic DNA from *S. aureus* and CoNS isolates was extracted using enzymatic lysis using the buffers and solutions provided with the *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany) and a DNeasy® Blood and Tissue kit (Qiagen, Crawley, UK). Genetic characterisation of isolates was undertaken by DNA microarray profiling using the *S. aureus* Genotyping Kit 2.0 as described previously [12 13]. The kit detects 333 gene targets including staphylococcal antimicrobial-resistance, virulence, SCC*mec* and ACME-*arc* genes and assigns *S. aureus* isolates to multilocus sequence type (ST) or clonal complexes (CC)s. MRSA phenotype was confirmed in *S. aureus* and CoNS isolates positive for *mecA* by growth of pink (*S. aureus*) or colorless/white (CoNS) colonies on MRSASelect agar (Bio-Rad®, Hercules, CA, USA). When required, confirmation of carriage of fusidic acid resistance genes *fusC, fusB*, and toxic shock syndrome toxin gene (*tst1*) were confirmed by PCR using the primers and conditions described by O'Neill *et al* [14] and Chen *et al* [15].

Fusidic acid susceptibility testing

Fusidic acid MICs were determined by ETEST® (bioMérieux, Marcy-l'Etoile, France) according to manufacturer's instructions. Thirty *S. aureus* and CoNS isolates harbouring *fus*C or *fus*B were sub-cultured twice before testing. Results were interpreted according to EUCAST (http://www.eucast.org, assessed May 2015) susceptibility criteria.

Statistical analyses

Fisher's exact test was used to analyze categorical variables (prevalence of genes) using GraphPad QuickCalcs on-line software. The significance of differences between

groups was expressed as two-tailed p-values, *p* values of ≤ 0.05 were considered statistically significant.

Results

Nasal carriage of staphylococcal species and regional distribution

Thirty-one percent (137/444) of students were positive for nasal carriage of *S. aureus* of whom 6.6 % (9/137) were MRSA. Eighty-seven percent (386/444) of students were positive for nasal carriage of CoNS (*S. epidermidis* (82% 364/444), *S. haemolyticus* (3% 14/444) or *S. saprophyticus* (2% 8/444) (Table 1). All students positive for *S. aureus* also carried *S. epidermidis.* Methicillin-resistant (MR)-CoNS were investigated in the *S. aureus*-positive cohort only of which 13.1 % (18/137) were MR-CoNS. One student exhibited co-carriage of MRSA and MR-CoNS. The geographical region of origin of students harbouring *S. aureus* and CoNS is shown in Figure 1. The Middle East, Europe and North America accounted for 68.6% of *S. aureus* carriers. For regions represented by ≥ 12 participants, the rate of nasal carriage of *S. aureus* varied geographically between 17 % (South East Asia) and 44 % (Africa).

Clonal lineages among *S. aureus* isolates

The ST or CC distribution among 137 *S. aureus* isolates is shown in Figure 2. Isolates belonged to a variety of CCs with 46/137 (33.5%) assigned to internationally disseminated CC5, CC8, CC22, CC30, CC45. A further 24/137 (17.5%) isolates belonged to CC1, CC59, CC88 or CC398.

SCC*mec* types and fusidic acid resistance among *S. aureus* and CoNS

Of the 333 staphylococcal genes detected by the microarray, the two most prevalent antibiotic resistance genes among nasal staphylococci were those encoding resistance to β-lactams and fusidic acid. The most common SCC*mec* type among nasal MRSA (n=9) and MR-CoNS (n=18) was SCC*mec* type IV (class B *mec* (*mecA, DmecR1, ugpQ*) and *ccrA-2*, *ccrB-2*). The nine MRSA isolates belonged to ST59-MRSA-IV (6/9) and CC1-MRSA-V-SCC*fus* (3/9) (class C *mec* (*mecA, ugpQ) and ccrC* and *fusC* (Q6GD50) and *ccrA-1, ccrB-1*). Among 18 MR-CoNS identified (17 *S. epidermidis* and 1 *S. saprophyticus*), half harboured SCC*mec* type IV (8 *S. epidermidis* and the single *S. saprophyticus*). SCC*mec* types II, V and VII were identified in three, five and one of the remaining *S. epidermidis* isolates, respectively. Isolates from the one individual who exhibited nasal co-carriage of MRSA and MR-CoNS (*S. epidermidis*) both harboured SCC*mec* type IV (Table 2).

In addition to the three CC1-MRSA-V isolates that carried SCC*fus*, the fusidic acid resistance genes *fusC* and *fusB* were identified in 28/128 (21.8%) and 2/128 (1.5%) of methicillin-susceptible *S. aureus* (MSSA) isolates, respectively. Ten of the 28 *fusC*-positive MSSA isolates belonged to CC1-MSSA-SCC*fus* and 18 were CC88-MSSA. All 10 CC1-MSSA-SCC*fus* isolates harboured a combination of SCC*fus* with the cassette chromosome recombinase (*ccr*) genes, *ccrA-1* and *ccrB-1*. The two *fus*B positive isolates belonged to CC5-MSSA and CC8-MSSA (Table 2). Among MR-CoNS, 27.7 % (5/18) *S. epidermidis* isolates carried *fus*C (two of them also carried *ccr* genes *ccrA*-1 *ccrB*-1) and 50 % *fusB* (9/18, eight *S. epidermidis* and the one *S. saprophyticus*). Among methicillin susceptible CoNS isolates, the *fus*C and *fus*B genes were identified in 20/119 (16.8%, 18 *S. epidermidis* and two *S. saprophyticus*) and 18/119 (15.1%, all *S. epidermidis*), respectively. One participant had nasal co-carriage of *fusC*-positive *S. aureus* (CC88-MSSA) and CoNS (*S. epidermidis*).

All SCC*mec* positive staphylococci were confirmed to have an MRSA/MR-CoNS phenotype. However, there was poor correlation between *fusC/fusB* carriage

and phenotypic fusidic acid resistance. Fusidic acid MICs for all *fusC* or *fusB*-positive *S. aureus* and CoNS isolates are shown in Table 3. Phenotypic fusidic acid resistance was confirmed (based on EUCAST breakpoints, MICs ≥1 μg/ml) in 23/32 (71.8%) *S. aureus* and 20/38 (52.6%) CoNS nasal isolates harbouring either *fus*C or *fus*B (DNA microarray result confirmed by PCR). Eight nasal isolates (three *S. aureus*, five *S. epidermidis*) positive for *fusB* exhibited high level fusidic acid resistance (MIC≥ 32 μg/ml). Fusidic acid resistance was inducible in a further three *S. aureus* and seven *S. epidermidis* isolates following incubation with 0.01 μg/ml fusidic acid BHI agar.

Other notable antimicrobial resistance genes among nasal *S. aureus* and CoNS

Apart from SCC*mec* element and *fus* genes, other antimicrobial genes detected among staphylococcal nasal flora were identified by DNA microarray. Tetracycline resistance genes, *tet(*K) or *tet(*M), were detected in 13/137 (9.5 %) of *S. aureus* isolates and 6/137 (4.3%) of the CoNS isolates. The quaternary ammonium compound resistance genes (*qac*A/*qac*C), encoding antiseptic resistance, were significantly more prevalent among CoNS isolates compared to *S. aureus* isolates (29/137 (21.2%) *Vs* 2/137 (1.4%), p<0.0001). Significantly more CoNS than *S. aureus* isolates carried *ileS2* encoding high-level mupirocin resistance (11/137 (8%) *vs* 1/137 (0.72%), p<0.01). However, none of these genes were common to *S. aureus*/CoNS pairs recovered from the same individual. The β-lactamase genes were abundant among *S. aureus* and CoNS; *bla*Z was present in 101/137 (73.72%) *S. aureus* isolates and 92/137 (67.1%) CoNS isolate and in 74/137 (54%) of individuals, these genes were common to *S. aureus*/CoNS pairs from the same nares. A summary of the antibiotic resistance genes found among *S. aureus* and CoNS is shown in Table 2. The staphylococcal isolates were negative for all other antibiotic resistance genes spotted on the microarray.

Virulence genes among nasal *S. aureus* and CoNS

A single isolate, CC30-MSSA, was positive for the Panton-Valentine leucocidin genes (*lukF/S-PV*). Among nasal staphylococci, ACME-*arc* was significantly associated with CoNS compared to *S. aureus* (44/137 (32.1%) Vs 1/137 (0.7%)), p<0.0001. The toxic shock syndrome toxin gene *tst1* was identified in 33/137 (24.1%) nasal *S. aureus* isolates. Unusually, DNA microarray identified *tst1* in two *S. epidermidis* isolates and this was confirmed by PCR. ACME-*arc* was common to *S. aureus*/CoNS recovered from the nares in one individual only. One hundred and two (74.4%) *S. aureus* isolates encoded one or more enterotoxin genes. The enterotoxin gene cluster (egc), containing *seg, sei, sem, sen, seo, seu*) was the most prevalent (48/102, 47%) followed by *seq/k* (13/102, 12.7 %) and *sec/l* (7/102, 6.8 %). The staphylococcal isolates were negative for all other toxin genes spotted on the microarray.

Discussion

Studies of staphylococcal carriage and epidemiology among the healthy population in the absence of significant antibiotic pressure are important in identifying the potential for pathogenic evolution. To our knowledge, this is the first study to co-investigate CoNS and *S. aureus* when recovered together from the nares of healthy pre-clinical medical students. The species distribution of nasal colonizing CoNS was similar to other studies [16] although the enrichment methods used here favoured *S. aureus* and *S. epidermidis* and may explain the low prevalence of other CoNS species. Our study revealed that, apart from the *bla* genes, which are abundant among staphylococci, the rates of co-carriage of antibiotic resistance genes in paired *S. aureus*/CoNS from the same individual were low in the community setting at <1%. Rates of simultaneous carriage of antimicrobial resistance among nasal staphylococci are likely to be higher under selective antibiotic pressure but few studies have investigated this among patients. One small study of hospitalized patients with nasal carriage of *S. aureus* and CoNS reported a rate of 12.5 % patients carrying MRSA and

MR-CoNS [17]. However, the authors reported only two cases where simultaneous carriage of MR-CoNS and MRSA was detected and the strains involved carried different SCC*mec* types. Despite negligible detection of co-species nasal carriage of these genes in medical students prior to healthcare exposure, based on antimicrobial resistance gene carriage by CoNS from this cohort, there is significant potential for mobilisation of genes to *S. aureus* that may enhance its pathogenic potential in the healthcare setting.

DNA microarray analyses revealed carriage of SCC*mec, fusC, fusB, ileS2, qacA/qacC* and ACME-*arc* among colonising nasal staphylococci in individuals with no previous healthcare exposure with greater prevalence among CoNS than *S. aureus*. This pattern among pre-clinical medical students, supports CoNS as a reservoir with potential to subsequently accelerate antimicrobial resistance and pathogenicity among colonizing *S. aureus* in clinical environments under antibiotic selective pressure [16 18 19].

Despite considerable geographical distribution of the participants in this study, a *S. aureus* nasal carriage rate in the community of 30.8% was recorded. In this study, CC30, CC88 and CC8 were the most prevalent clones identified among nasal *S. aureus*. CC30 is among the internationally disseminated clones in which SCC*mec* has been acquired and is a successful colonising lineage, reported among HA and CA-MRSA. Among medical students, these MSSA isolates may therefore represent a significant pool for the uptake of SCC*mec* in a clinical setting. CC88 is frequently isolated in Australia but in our study the geographical background of isolates was mixed (including Middle East, Europe, South East Asia and Central America). CC8 is associated with MRSA infection and is globally disseminated [20]. Although CC30, CC88 and CC8 were prevalent among community MSSA isolates in this study, among the relatively few MRSA recovered, none belonged to these CCs. Two CC/ST types detected among MRSA recovered from healthy medical students in this study were ST59-MRSA-IV and CC1-MRSA-V-SCC*fus*C. ST59 (Western Australian-MRSA-73) is

a sporadic Australian strain and apart from PVL-negativity, is indistinguishable from USA1000 [21] In this study the geographical background of these isolates was wide (Middle East, North America and South East Asia).

The identification of a significant reservoir of antibiotic resistance among medical students prior to healthcare exposure in subsequent clinical years, highlights the need for effective infection prevention and control policies in relation to hand hygiene and surveillance. In the absence of antibiotic selective pressure, the colonising MRSA rate appears relatively stable and in this study was 2 % (9/444), similar to rates reported elsewhere [22]. However, a previous study among medical interns in China reported a nasal MRSA rate of 9.4% likely reflecting exposure to the healthcare environment [23]. One study reported an increasing in carriage rates of MR-CoNS from 14% among medical student pre-internship, to 29.28% among interns [24]. Prevalence rates of MR-CoNS in recent community-based surveys are variable but rates of 16.5% [19] and 17.2% [25] are reported in similar cohorts to this study where, of those colonised with *S. aureus*, 13.1% carried MR-CoNS. SCC*mec* type IV, the smallest of the SCC*mec* elements, was the most prevalent type among MRSA and MR-CoNS here (66.6% and 50%). SCC*mec* IV has been detected in approximately 40% of methicillin-resistant *S. epidermidis* identified in humans [26]. However, in this study SCC*mec* type V was also represented among MRSA and MR-CoNS. While only one individual was colonised with MRSA and MR-CoNS in this study (both SCC*mec* type IV), the preponderance of SCC*mec* IV element in nasal MRSA and MR-CoNS suggests the potential for *mec*A gene transfer among these species even in the absence of selective pressure. The small size of this element, which has a low fitness cost, may enhance its dissemination potential. [27]

Fusidic acid resistance among *S. aureus* from healthy carriers in nine European countries in 2014 was reported to be <10 % [28]. However, we found a prevalence of 22.6% of *fusC/fusB* genes among healthy carriers. Fusidic acid resistance appears to

correlate with increased use of this agent. For example, in New Zealand, where it is used as a first-line empiric agent for topical treatment of impetigo, prevalence rates of resistance in community *S. aureus* isolates increased from 17% in 1999 to 29% in 2013 [29]. In Europe, fusidic acid is combined with β-lactams for the treatment of staphylococcal bacteraemia, endocarditis, and osteomyelitis [30] and is used widely in the community for skin and soft tissue infections (SSTIs). A 2010 study of fusidic acid resistance among *S. aureus* clinical isolates showed Greece and Ireland to have the highest rates (52.5 and 19.9%) [31]. SCC*fus* has been identified in the CC1 background and more recently, in other lineages such as ST239 and ST779 [32-34]. As highlighted here in the absence of significant antibiotic pressure in the community, it appears that this element is associated with MRSA and MSSA in the CC1 background. This genetic platform, particularly when associated with SCC*mec* in a composite element (SCC*mec* V+SCC*fus*) may enable the transfer of multi-drug resistance in a single transfer event. The use of fusidic acid is un-regulated in some countries and hence it may be used inappropriately in a community setting (for example in short or discontinuous doses). Inappropriate use of fusidic acid may therefore favour co-selection of methicillin-resistance among *S. aureus*. In addition, in this study, 14/18 (77%) of MR-CoNS were positive for *fusC* or *fusB*. This association of resistances among the resident flora may provide further opportunity for dissemination of MRSA driven by fusidic acid selective pressure. Interestingly a positive correlation between carriage of *fusC/fusB* and phenotypic resistance was observed in only 71.9 % and 53.6 % of *S. aureus* and CoNS respectively. However, induction of gene expression with fusidic acid pre-incubation gave better correlation (82.2% and 76.3 % correlation).

There were limitations to this study, which included; a single centred, relatively small study. Some nasally abundant CoNS species, for example *Staphylococcus lugdunensis* and *Staphylococcus hominis,* were under-represented as the enrichment method favoured pathogenic staphylococci such as *S. aureus* and *S. epidermidis*.

CoNS was investigated only in those co-colonised with *S. aureus* and therefore prevalence rates for genes among CoNS do not reflect the entire cohort. CCs and STs were determined only among *S. aureus* as the high rate of genetic recombination among CoNS makes strain typing unreliable. Although the microarray system used is reported effective for staphylococcal species other than *S. aureus* [35], some gene targets may be heterologous among staphylococci leading to false negatives. The study design did not facilitate follow-up of this cohort during clinical training which may have revealed further changes in gene carriage among colonising staphylococci. However, the multi-national origin of the student body in our institution facilitated analysis of a relatively broad geographic cohort in a single study and emphasises the role that importation plays in *S. aureus* epidemiology. Unlike other studies of staphylococci in the healthy human nares, pairs of staphylococcal species originating from the same individual were investigated here for their resistance and virulence traits. These data support a low rate of transfer of antibiotic resistance between colonising staphylococcal species in the absence of healthcare contact. However, it is concerning that similar SCC*mec* and SCC*fus*C types, in addition to *ileS2, qacA/qacB* and ACME are carried among CoNS and *S. aureus* in healthy individuals who will have subsequent roles in healthcare provision. Given the increasing emergence of HA-MRSA with features of community strains, further mobilisation of these elements under selective antibiotic pressure may enhance the transmission and success of *S. aureus* in the healthcare environment.

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Competing interests

HH has received funding from Pfizer and Astellas outside the relevance of the submitted work, all other authors report no competing interests.

Contributor Statement

PEB and DFH recruited students to the study, PEB conducted the laboratory work and drafted the manuscript. DFH and HH conceived of the study and contributed to study design. AS, PK and DC provided critical data interpretation and revised the drafted work. All authors contributed to the final approved draft.

Data sharing statement

All data for these analyses are included in the manuscript. No additional data are available.

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Figure Legends

Figure 1. **Geographical origin of medical students recruited**. The geographical areas of origin of 444 medical students recruited to the study are shown (dark grey bars). Of those recruited, 137 were confirmed nasal *S. aureus* and CoNS positive. The proportion of recruited students from each geographical origin with nasal *S. aureus* carriage are also shown (light grey bars).

Figure 2. Genotypic diversity of 137 *S. aureus* nasal isolates using DNA microarray analysis, including 128 MSSA (dark grey bars) and 9 MRSA (light grey bars). Letter (a)

indicates internationally disseminated clones into which SCC*mec* can integrate. CC=clonal complex.

Table 1. Staphylococci species recovered from 444 nasal swabs.

Staphylococcal species recovered	Total n, % n=444	Methicillin-resistant phenotype n,% n=137[a]
S. aureus	137, 30.8	
MRSA		9, 6.6
S. epidermidis	364, 81.9	
MRSE		17, 12.4
S. haemolyticus	14, 3.1	
MRSH		0
S. saprophyticus	8, 1.8	
MRSS		1, 0.72
Co-carriage species		
S. aureus + *S. epidermidis*	137, 30.8	
MRSA + MR-CoNS		1, 0.72
fusC positive *S. aureus* + CoNS		1, 0.72

[a] CoNS were investigated only in those positive for nasal *S.*

aureus in the student cohort and not in all those recruited. MRSA= methicillin resistant *S. aureus*, MRSE + methicillin resistant *S. epidermidis*, MRSH = methicillin resistant *S. haemolyticus*, MRSS = methicillin resistant *S. saprophyticus*

411 Table 2. Resistance/virulence genes detected among 137 co-located nasal *S. aureus*/CoNS pairs.

Detected Gene(s)	**Phenotypic resistance/trait**	**No. isolates positive n (%)**			**No. *S. aureus*/CoNS pairs positive (n)**
		S. aureus **n=137**	**CoNS n=137**	***P* value**	
Antibiotic resistance gene					
blaZ	β-lactam	101 (73.7)	92 (67.1)	0.289	74
fusB	Fusidic acid	2 (1.5)	27 (19.7)	0.0002*	0
fusC[a]	Fusidic acid	31 (22.6)	25 (18.2)	0.159	1
mecA	Methicillin	9 (6.5)	18 (13.1)	0.103	1
ileS2	Mupirocin	1 (0.7)	11 (8.0)	0.005*	1
*qac*A and *qac*C	Quartenary ammonium salts	3 (2.2)	29 (21.2)	<0.0001*	0
tet(K) and tet(M)	Tetracycline	13 (9.5)	6 (4.4)	0.152	0
erm(C)	Macrolide/lincosamide	6 (4.3)	5 (3.6)	1.000	0
msr(A)	Macrolide	2 (1.45)	15 (10.9)	0.002*	1
mph(C)	Macrolide	0	15 (10.9)	<0.0001*	0
dfrS1	Trimethoprim	0	19 (13.8)	<0.0001*	0

vga	Streptogramin A	1 (0.7)	6 (4.3)	0.120	0
Virulence					
ACME-*arc*	pH tolerance	1 (0.7)	44 (32.1)	<0.0001*	1
tst1[b]	Toxic shock toxin	33 (24.1)	2 (1.5)	<0.0001*	0

[a]associated with SCC element, (*ccrA-1* and *ccrB-1*) in 13/137 *S. aureus*. [b]*tst1* confirmed by PCR. ACME = Arginine Catabolite Mobile Element. * indicates a statistically significant result by Fisher's exact test.

Table 3. Fusidic acid MICs for *S. aureus* and CoNS

MIC Interpretation[a]	MIC ≤ 1 µg/ml S n (%)	MIC ≥ 1 µg/ml R n(%)	MIC ≥ 32 µg/ml HR n (%)
***S. aureus* (n = 32)**	9 (28.1)	20 (62.5)	3 (9.3)
CoNS (n = 38)	18 (47.4)	15 (39.5)	5 (13.2)

[a]Interpretation based on The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1, 2017. http://www.eucast.org, S = Susceptible, R = Resistant, HR = high level resistant

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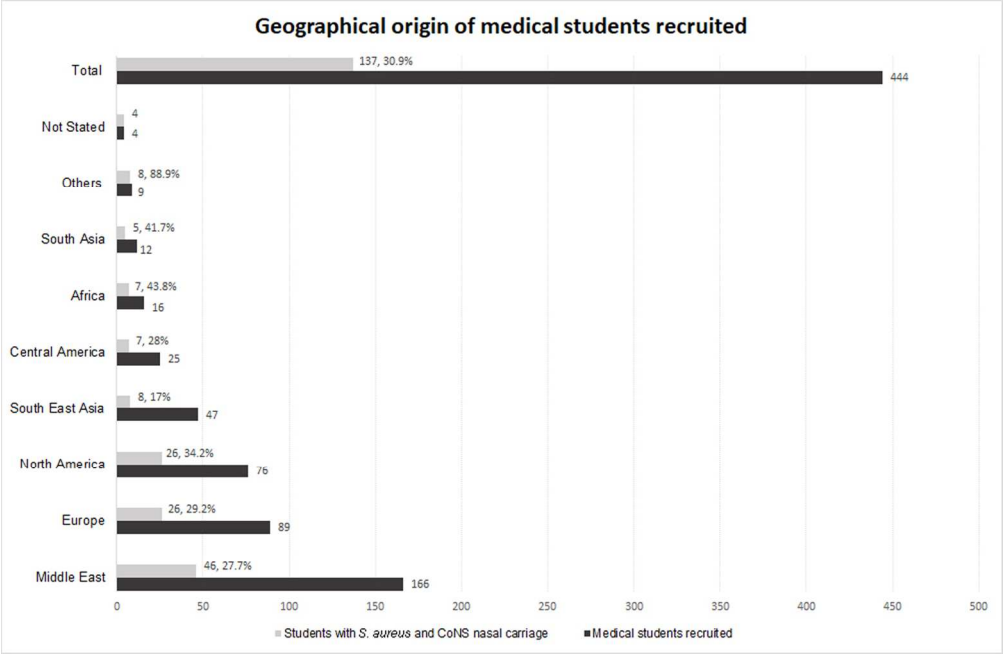
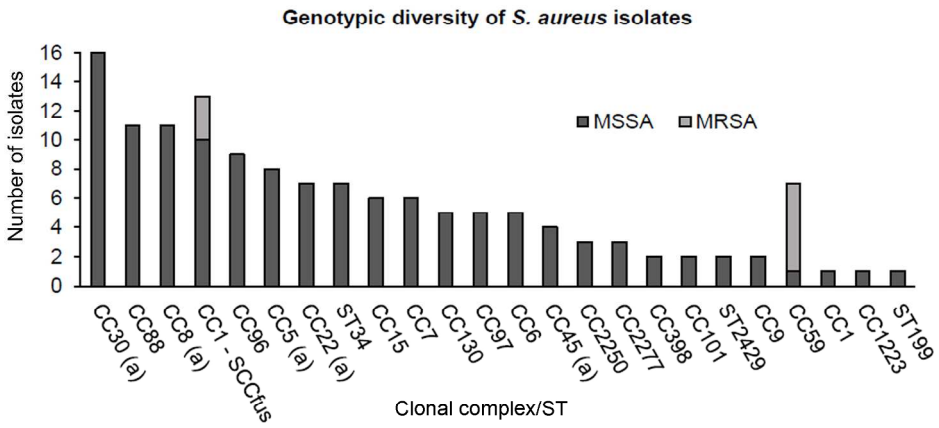


Figure 1. Geographical origin of medical students recruited. The geographical areas of origin of 444 medical students recruited to the study are shown (dark grey bars). Of those recruited, 137 were confirmed nasal *S. aureus* and CoNS positive. The proportion of recruited students from each geographical origin with nasal S. aureus carriage are also shown (light grey bars).

147x95mm (300 x 300 DPI)



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222x127mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Relevent lines in manuscript and confirmation of items
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1- Title 1-Abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Yes
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Yes
Objectives	3	State specific objectives, including any prespecified hypotheses	25-29 (Abstract) 93-97 (Introduction)
Methods			
Study design	4	Present key elements of study design early in the paper	100
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	99-113
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	103-106
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	n/a n/a
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	n/a
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	134-138 (Identification of genes) 146-149 (fusidic acid susceptibility)

			110-111 (participant details, age range, region of orignin, healthcare exposure)
Bias	9	Describe any efforts to address potential sources of bias	102-106 (anonymous data collection, high participation rate no healthcare contact reported etc)
Study size	10	Explain how the study size was arrived at	105
Quantitative variables	11	Explain how quantitative variables werehandled in the analyses. If applicable, describe which groupings were chosen and why	152-154 (statistical analyses of variables)
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	152-154
		(*b*) Describe any methods used to examine subgroups and interactions	n/a
		(*c*) Explain how missing data were addressed	n/a
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	n/a n/a n/a
		(*e*) Describe any sensitivity analyses	

Continued on next page

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	105, 158
		(b) Give reasons for non-participation at each stage	n/a
		(c) Consider use of a flow diagram	n/a
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	164-165 (relevant demographic region of origin)
		(b) Indicate number of participants with missing data for each variable of interest	n/a
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	n/a
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	*n/a*
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	*n/a*
		Cross-sectional study—Report numbers of outcome events or summary measures	*158,159 S. aureus and MRSA colonisation* *162-163 MRSE among S. aureus colonised* *163-163 Co-carriage of MRSA MRSE*
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	179-183 SCCmec types among MRSA 183-186 SCCmec types among CoNS 189-194 fusC carriage among S. aureus and CoNS 200-201 Co-carriage of fus C 205-209 fusidic acid resistance among S. aureus/CoNS Confounders – n/a
		(*b*) Report category boundaries when continuous variables were categorized	n/a
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	n/a
Discussion			
Key results	18	Summarise key results with reference to study objectives	249-254 257-260 285-288 300-303
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both	330-341

		direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	YES
Generalisability	21	Discuss the generalisability (external validity) of the study results	285-290 – comparison to other studies, stability of findings in relations to carriage rates
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	356-358

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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An observational cross-sectional study of nasal staphylococcal species of medical students of diverse geographical origin, prior to healthcare exposure. Prevalence of SCC*mec*, *fusC*, *fusB* and the arginine catabolite mobile element (ACME) in the absence of selective antibiotic pressure.

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An observational cross-sectional study of nasal staphylococcal species of medical students of diverse geographical origin, prior to healthcare exposure. Prevalence of SCC*mec*, *fusC*, *fusB* and the arginine catabolite mobile element (ACME) in the absence of selective antibiotic pressure.

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Running title: Antimicrobial resistance and virulence genes among staphylococcal carriage isolates.

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Abstract

Objective: The aim of this study was to investigate co-located nasal *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) (mainly *Staphylococcus epidermidis*), recovered from healthy medical students in their pre-clinical year, prior to exposure to the healthcare environment, for the carriage of genes and genetic elements common to both species and that may contribute to *S. aureus* and methicillin-resistant *S. aureus* (MRSA) evolution.

Design: Prospective observational cross-sectional study. Carriage of antimicrobial resistance and virulence-associated genes in the absence of significant antibiotic selective pressure was investigated among healthy medical students from geographically diverse origins who were nasally co-colonised with *S. aureus* and CoNS. Clonal lineages of *S. aureus* isolates were determined.

Setting/Participants: Dublin-based international undergraduate medical students

Results: Nasal *S. aureus* carriage was identified in 137/444 (30.8%) students of whom nine (6.6%) carried MRSA (ST59-MRSA-IV (6/9), CC1-MRSA-V-SCC*fus* (3/9)). The genes *mecA*, *fusB*, *ileS2, qacA*/*qacC* and the arginine catabolite mobile element (ACME)-*arc* were detected among colonizing nasal staphylococci and had a significantly greater association with CoNS than *S. aureus.* The rate of co-carriage of any of these genes in *S. aureus*/CoNS pairs recovered from the same individual was <1 %.

Conclusions The relatively high prevalence of these genes among CoNS of the healthy human flora in the absence of significant antibiotic selective pressure is of interest. Further research is required to determine what factors are involved and whether these are modifiable to help prevent the emergence and spread of antibiotic resistance amongst staphylococci.

Strengths and limitations of this study:

- Global evaluation of antibiotic resistance gene carriage among Staphylococci among healthy medical students in preclinical years through DNA microarray analyses.
- Pairs of staphylococcal species were isolated from the same colonisation site (nares) of multiple participants to allow investigation of shared antibiotic resistance and virulence in the same human niche in a community setting.
- A single centre study design.
- CoNS was investigated only in students co-colonised with *S. aureus*.
- The study design did not facilitate follow-up of this cohort during clinical training.

Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* are significant colonisers of healthy human skin and nares and are among the leading causes of healthcare-associated infection (HAI). Morbidity, mortality and the financial burden associated with methicillin-resistant *S. aureus* (MRSA) infections are well documented. Furthermore, coagulase-negative staphylococci (CoNS) including *S. epidermidis* are reported reservoirs of antimicrobial-resistance genes and their associated mobile genetic elements, most notably the staphylococcal cassette chromosome (SCC) harbouring the *mec* gene (SCC*mec*) [1].

Twelve SCC*mec* types and numerous subtypes have been described among MRSA isolates to date. The more prevalent and diverse range of SCCs and SCC*mec* among CoNS further supports CoNS as a reservoir for antimicrobial resistance genes [1]. The identification of SCC, SCC*mec* and SCC-associated elements with other antimicrobial and virulence genes and their epidemiological relationships among clinical staphylococci has advanced our understanding of the role of CoNS in the

evolution of MRSA [2]. For example, the fusidic acid resistance gene *fusC* is associated with SCC*mec* IV-SCC_{476} and other SCC-like elements have been identified in *S. aureus*, MRSA and CoNS and may contribute to MRSA emergence in countries with significant fusidic acid usage. [3-5] Furthermore, the SCC-like arginine catabolic mobile element (ACME) which enhances acid tolerance, is abundant among clinical CoNS isolates, in particular *S. epidermidis* and *S. haemolyticus* [6]. Among *S. aureus,* ACME has mainly been detected among isolates of the community-associated (CA) USA300 clone [7]. CoNS are also a putative reservoir of the high level mupirocin resistance encoding gene *ileS2*, which is also increasing among *S. aureus*/MRSA in healthcare and community environments related to horizontal gene transfer or expansion of specific clones [8 9].

Increasingly, MRSA clones previously associated with the community, such as clonal complex (CC) 1 are spreading to healthcare settings making the differentiation between healthcare-associated (HA) MRSA and CA-MRSA unclear [10]. Therefore, detailed investigation of the genetic and phenotypic traits of colonizing staphylococcal species in community settings are important to identify those with features that may contribute to their evolution into potentially successful and formidable healthcare-associated clones. The aim of this study was to investigate co-located nasal *S. aureus* and CoNS (mainly *S. epidermidis*) recovered from healthy medical students in their pre-clinical year, prior to exposure to the healthcare environment, for the carriage of genes and genetic elements common to both species and that may contribute to *S. aureus* and MRSA evolution.

Methods

Study setting, participants and sample collection

This observational cross-sectional study was conducted at the Royal College of Surgeons in Ireland (RCSI) from December 2014 – January 2016. Nasal swabs

(eSwab Copan®), Italy) were collected anonymously from undergraduate medical students. Eligible students were those attending the RCSI medical centre to submit a swab for mandatory MRSA screening in the week before they began their clinical attachments. In total 444/450 eligible medical students (250 (56.3%) male, 194 (43.7%) female) participated in this study. All participants reported no previous hospital contact in the six weeks prior to recruitment. The student volunteers were from the second year of the undergraduate medical programme and as such, all participants were domiciled in Ireland for a minimum of two years prior to recruitment. Data was collected anonymously from each participant, including age range, region of origin and previous healthcare contact. Ethical approval (approval number REC949) was obtained from the Institute's Ethics Committee and informed consent was obtained from each participant.

Sample preparation

Swabs were processed to recover *S. aureus* (including MRSA) and pathogenic CoNS species using a modification of a published method[11]. Swabs were enriched in brain heart infusion (BHI) supplemented with 6% (w/v) NaCl for 24 h at 37°C followed by further enrichment in mannitol salt broth for 24 h at 37°C. The enriched culture was diluted 1/1000 and 100 µl was spread onto SaSelect agar (Bio-Rad®, Hercules, CA, USA). Plates that yielded pink/orange colonies (presumptive *S. aureus*) were inspected for growth of colonies of relevant CoNS based on colony colour (e.g. light pink colonies of various sizes, presumptive *S. epidermidis*; white/yellow colonies, *S. haemolyticus*, *S. hominis*, *S. capitis*, *S. warneri*, *S. caprae*, *S. lugdunensis*). Presumptive CoNS species and *S. aureus* were sub-cultured from these plates onto Columbia blood agar (CBA) and identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF) using a MALDI Biotyper (Microflex LT, Bruker). Matched isolates (where *S. aureus* and a CoNS species were recovered from the same swab)

were cryopreserved and stored at -20°C (Protect™ bacterial preserver beads (Technical Service Consultants, UK).

Characterisation of *S. aureus* and CoNS isolates

Genomic DNA from *S. aureus* and CoNS isolates was extracted using enzymatic lysis using the buffers and solutions provided with the *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany) and a DNeasy® Blood and Tissue kit (Qiagen, Crawley, UK). Genetic characterisation of isolates was undertaken by DNA microarray profiling using the *S. aureus* Genotyping Kit 2.0 as described previously [12 13]. The kit detects 333 gene targets including staphylococcal antimicrobial-resistance, virulence, SCC*mec* and ACME-*arc* genes and assigns *S. aureus* isolates to multilocus sequence type (ST) or clonal complexes (CC)s. MRSA phenotype was confirmed in *S. aureus* and CoNS isolates positive for *mecA* by growth of pink (*S. aureus*) or colorless/white (CoNS) colonies on MRSASelect agar (Bio-Rad®, Hercules, CA, USA). When required, confirmation of carriage of fusidic acid resistance genes *fusC, fusB*, and toxic shock syndrome toxin gene (*tst1*) were confirmed by PCR using the primers and conditions described by O'Neill *et al* [14] and Chen *et al* [15].

Fusidic acid susceptibility testing

Fusidic acid MICs were determined by ETEST® (bioMérieux, Marcy-l'Etoile, France) according to manufacturer's instructions. Thirty *S. aureus* and CoNS isolates harbouring *fus*C or *fus*B were sub-cultured twice before testing. Results were interpreted according to EUCAST (http://www.eucast.org, assessed May 2015) susceptibility criteria.

Statistical analyses

Fisher's exact test was used to analyze categorical variables (prevalence of genes) using GraphPad QuickCalcs on-line software. The significance of differences between groups was expressed as two-tailed p-values, *p* values of ≤ 0.05 were considered statistically significant.

Patient and Public Involvement statement:

The participants in this study were medical undergraduate students. They were invited to participate in this study when they underwent mandatory MRSA screening prior to commencement of clinical placements. As future clinicians, participants were broadly considered in the development of the research question. As nasal carriage of *S. aureus* and MRSA among healthcare staff contributes to transmission to patients in healthcare environments, medical students have an interest in contributing to this knowledge. Participants did not contribute to the study design. Participants were informed at recruitment that dissemination of the results would be through the scientific literature.

Results

Nasal carriage of staphylococcal species and regional distribution

Thirty-one percent (137/444) of students were positive for nasal carriage of *S. aureus* of whom 6.6 % (9/137) were MRSA. Eighty-seven percent (386/444) of students were positive for nasal carriage of CoNS (*S. epidermidis* (82% 364/444), *S. haemolyticus* (3% 14/444) or *S. saprophyticus* (2% 8/444) (Table 1). All students positive for *S. aureus* also carried *S. epidermidis.* Methicillin-resistant (MR)-CoNS were investigated in the *S. aureus*-positive cohort only of which 13.1 % (18/137) were MR-CoNS. One student exhibited co-carriage of MRSA and MR-CoNS. The geographical region of origin of students harbouring *S. aureus* and CoNS is shown in Figure 1. The Middle East, Europe and North America accounted for 68.6% of *S. aureus* carriers. For regions represented by ≥ 12 participants, the rate of nasal carriage of *S. aureus* varied geographically between 17 % (South East Asia) and 44 % (Africa).

182

183 **Clonal lineages among *S. aureus* isolates**

184 The ST or CC distribution among 137 *S. aureus* isolates is shown in Figure 2. Isolates
185 belonged to a variety of CCs with 46/137 (33.5%) assigned to internationally
186 disseminated CC5, CC8, CC22, CC30, CC45. A further 24/137 (17.5%) isolates
187 belonged to CC1, CC59, CC88 or CC398.

188

189 **SCC*mec* types and fusidic acid resistance among *S. aureus* and CoNS**

190 Of the 333 staphylococcal genes detected by the microarray, the two most prevalent
191 antibiotic resistance genes among nasal staphylococci were those encoding resistance
192 to β-lactams and fusidic acid. The most common SCC*mec* type among nasal MRSA
193 (n=9) and MR-CoNS (n=18) was SCC*mec* type IV (class B *mec* (*mecA, DmecR1,*
194 *ugpQ*) and *ccrA-2*, *ccrB-2*). The nine MRSA isolates belonged to ST59-MRSA-IV (6/9)
195 and CC1-MRSA-V-SCC*fus* (3/9) (class C *mec* (*mecA, ugpQ) and ccrC* and *fusC*
196 (Q6GD50) and cassette chromosome recombinase (*ccr*) *A-1, ccrB-1*). Among 18 MR-
197 CoNS identified (17 *S. epidermidis* and 1 *S. saprophyticus*), half harboured SCC*mec*
198 type IV (8 *S. epidermidis* and the single *S. saprophyticus*). SCC*mec* types II, V and VII
199 were identified in three, five and one of the remaining *S. epidermidis* isolates,
200 respectively. Isolates from the one individual who exhibited nasal co-carriage of MRSA
201 and MR-CoNS (*S. epidermidis*) both harboured SCC*mec* type IV (Table 2).

202 In addition to the three CC1-MRSA-V isolates that carried SCC*fus*, the fusidic acid
203 resistance genes *fusC* and *fusB* were identified in 28/128 (21.8%) and 2/128 (1.5%) of
204 methicillin-susceptible *S. aureus* (MSSA) isolates, respectively. Ten of the 28 *fusC*-
205 positive MSSA isolates belonged to CC1-MSSA-SCC*fus*, 11 were CC88-MSSA and 7
206 CC8-MSSA. All 10 CC1-MSSA-SCC*fus* isolates harboured a combination of SCC*fus*
207 with the *ccr* genes, *ccrA-1* and *ccrB-1*. The two *fus*B positive isolates belonged to CC5-

MSSA and CC8-MSSA (Table 2). Among MR-CoNS, 27.7 % (5/18) *S. epidermidis* isolates carried *fus*C (two of them also carried *ccr* genes *ccrA*-1 *ccrB*-1) and 50 % *fusB* (9/18, eight *S. epidermidis* and the one *S. saprophyticus*). Among methicillin susceptible CoNS isolates, the *fus*C and *fus*B genes were identified in 20/119 (16.8%, 18 *S. epidermidis* and two *S. saprophyticus*) and 18/119 (15.1%, all *S. epidermidis*), respectively. One participant had nasal co-carriage of *fusC*-positive *S. aureus* (CC88-MSSA) and CoNS (*S. epidermidis*).

All SCC*mec* positive staphylococci were confirmed to have an MRSA/MR-CoNS phenotype. However, there was poor correlation between *fusC/fusB* carriage and phenotypic fusidic acid resistance. Fusidic acid MICs for all *fusC* or *fusB*-positive *S. aureus* and CoNS isolates are shown in Table 3. Phenotypic fusidic acid resistance was confirmed (based on EUCAST breakpoints, MICs ≥1 μg/ml) in 23/32 (71.8%) *S. aureus* and 20/38 (52.6%) CoNS nasal isolates harbouring either *fus*C or *fus*B (DNA microarray result confirmed by PCR). Eight nasal isolates (three *S. aureus*, five *S. epidermidis*) positive for *fusB* exhibited high level fusidic acid resistance (MIC≥ 32 μg/ml). Fusidic acid resistance was inducible in a further three *S. aureus* and seven *S. epidermidis* isolates following incubation with 0.01 μg/ml fusidic acid BHI agar.

Other notable antimicrobial resistance genes among nasal *S. aureus* and CoNS

Apart from SCC*mec* element and *fus* genes, other antimicrobial genes detected among staphylococcal nasal flora were identified by DNA microarray. Tetracycline resistance genes, *tet(*K) or *tet(*M), were detected in 13/137 (9.5 %) of *S. aureus* isolates and 6/137 (4.3%) of the CoNS isolates. The quaternary ammonium compound resistance genes (*qac*A/*qac*C), encoding antiseptic resistance, were significantly more prevalent among CoNS isolates compared to *S. aureus* isolates (29/137 (21.2%) *Vs* 2/137 (1.4%), p<0.0001). Significantly more CoNS than *S. aureus* isolates carried *ileS2* encoding high-level mupirocin resistance (11/137 (8%) *vs* 1/137 (0.72%), p<0.01). However, none of these genes were common to *S. aureus*/CoNS pairs recovered from the same

individual. The β-lactamase genes were abundant among *S. aureus* and CoNS; *blaZ* was present in 101/137 (73.72%) *S. aureus* isolates and 92/137 (67.1%) CoNS isolate and in 74/137 (54%) of individuals, these genes were common to *S. aureus*/CoNS pairs from the same nares. A summary of the antibiotic resistance genes found among *S. aureus* and CoNS is shown in Table 2. The staphylococcal isolates were negative for all other antibiotic resistance genes spotted on the microarray.

Virulence genes among nasal *S. aureus* and CoNS

A single isolate, CC30-MSSA, was positive for the Panton-Valentine leucocidin genes (*lukF/S-PV*). Among nasal staphylococci, ACME-*arc* was significantly associated with CoNS compared to *S. aureus* (44/137 (32.1%) Vs 1/137 (0.7%)), p<0.0001. The toxic shock syndrome toxin gene *tst1* was identified in 33/137 (24.1%) nasal *S. aureus* isolates. Unusually, DNA microarray identified *tst1* in two *S. epidermidis* isolates and this was confirmed by PCR. ACME-*arc* was common to *S. aureus*/CoNS recovered from the nares in one individual only. One hundred and two (74.4%) *S. aureus* isolates encoded one or more enterotoxin genes. The enterotoxin gene cluster (egc), containing *seg, sei, sem, sen, seo, seu*) was the most prevalent (48/102, 47%) followed by *seq/k* (13/102, 12.7 %) and *sec/l* (7/102, 6.8 %). The staphylococcal isolates were negative for all other toxin genes spotted on the microarray.

Discussion

Studies of staphylococcal carriage and epidemiology among the healthy population in the absence of significant antibiotic pressure are important in identifying the potential for pathogenic evolution. To our knowledge, this is the first study to co-investigate CoNS and *S. aureus* when recovered together from the nares of healthy pre-clinical medical students. The species distribution of nasal colonizing CoNS was similar to other studies [16] although the enrichment methods used here favoured *S.*

aureus and *S. epidermidis* and may explain the low prevalence of other CoNS species. Our study revealed that, apart from the *bla* genes, which are abundant among staphylococci, the rates of co-carriage of antibiotic resistance genes in paired *S. aureus*/CoNS from the same individual were low in the community setting at <1%. Rates of simultaneous carriage of antimicrobial resistance among nasal staphylococci are likely to be higher under selective antibiotic pressure but few studies have investigated this among patients. One small study of hospitalized patients with nasal carriage of *S. aureus* and CoNS reported a rate of 12.5 % patients carrying MRSA and MR-CoNS [17]. However, the authors reported only two cases where simultaneous carriage of MR-CoNS and MRSA was detected and the strains involved carried different SCC*mec* types. Despite negligible detection of co-species nasal carriage of these genes in medical students prior to healthcare exposure, based on antimicrobial resistance gene carriage by CoNS from this cohort, there is significant potential for mobilisation of genes to *S. aureus* that may enhance its pathogenic potential in the healthcare setting.

DNA microarray analyses revealed carriage of SCC*mec, fusC, fusB, ileS2, qacA/qacC* and ACME-*arc* among colonising nasal staphylococci in individuals with no previous healthcare exposure with greater prevalence among CoNS than *S. aureus*. This pattern among pre-clinical medical students, supports CoNS as a reservoir with potential to subsequently accelerate antimicrobial resistance and pathogenicity among colonizing *S. aureus* in clinical environments under antibiotic selective pressure [16 18 19].

Despite considerable geographical distribution of the participants in this study, a *S. aureus* nasal carriage rate in the community of 30.8% was recorded. In this study, CC30, CC88 and CC8 were the most prevalent clones identified among nasal *S. aureus*. CC30 is among the internationally disseminated clones in which SCC*mec* has been acquired and is a successful colonising lineage, reported among HA and CA-MRSA. Among medical students, these MSSA isolates may therefore represent a

significant pool for the uptake of SCC*mec* in a clinical setting. CC88 is frequently isolated in Australia but in our study the geographical background of isolates was mixed (including Middle East, Europe, South East Asia and Central America). CC8 is associated with MRSA infection and is globally disseminated [20]. Although CC30, CC88 and CC8 were prevalent among community MSSA isolates in this study, among the relatively few MRSA recovered, none belonged to these CCs. Two CC/ST types detected among MRSA recovered from healthy medical students in this study were ST59-MRSA-IV and CC1-MRSA-V-SCC*fus*C. ST59 (Western Australian-MRSA-73) is a sporadic Australian strain and apart from PVL-negativity, is indistinguishable from USA1000 [21] In this study the geographical background of these isolates was wide (Middle East, North America and South East Asia).

The identification of a significant reservoir of antibiotic resistance among medical students prior to healthcare exposure in subsequent clinical years, highlights the need for effective infection prevention and control policies in relation to hand hygiene and surveillance. In the absence of antibiotic selective pressure, the colonising MRSA rate appears relatively stable and in this study was 2 % (9/444), similar to rates reported elsewhere [22]. However, a previous study among medical interns in China reported a nasal MRSA rate of 9.4% likely reflecting exposure to the healthcare environment [23]. One study reported an increasing in carriage rates of MR-CoNS from 14% among medical student pre-internship, to 29.28% among interns [24]. Prevalence rates of MR-CoNS in recent community-based surveys are variable but rates of 16.5% [19] and 17.2% [25] are reported in similar cohorts to this study where, of those colonised with *S. aureus*, 13.1% carried MR-CoNS. SCC*mec* type IV, the smallest of the SCC*mec* elements, was the most prevalent type among MRSA and MR-CoNS here (66.6% and 50%). SCC*mec* IV has been detected in approximately 40% of methicillin-resistant *S. epidermidis* identified in humans [26]. However, in this study SCC*mec* type V was also represented among MRSA and MR-CoNS. While only one individual was

colonised with MRSA and MR-CoNS in this study (both SCC*mec* type IV), the preponderance of SCC*mec* IV element in nasal MRSA and MR-CoNS suggests the potential for *mec*A gene transfer among these species even in the absence of selective pressure. The small size of this element, which has a low fitness cost, may enhance its dissemination potential. [27]

Fusidic acid resistance among *S. aureus* from healthy carriers in nine European countries in 2014 was reported to be <10 % [28]. However, we found a prevalence of 22.6% of *fusC/fusB* genes among healthy carriers. Fusidic acid resistance appears to correlate with increased use of this agent. For example, in New Zealand, where it is used as a first-line empiric agent for topical treatment of impetigo, prevalence rates of resistance in community *S. aureus* isolates increased from 17% in 1999 to 29% in 2013 [29]. In Europe, fusidic acid is combined with β-lactams for the treatment of staphylococcal bacteraemia, endocarditis, and osteomyelitis [30] and is used widely in the community for skin and soft tissue infections (SSTIs). A 2010 study of fusidic acid resistance among *S. aureus* clinical isolates showed Greece and Ireland to have the highest rates (52.5 and 19.9%) [31]. SCC*fus* has been identified in the CC1 background and more recently, in other lineages such as ST239 and ST779 [32-34]. As highlighted here in the absence of significant antibiotic pressure in the community, it appears that this element is associated with MRSA and MSSA in the CC1 background. This genetic platform, particularly when associated with SCC*mec* in a composite element (SCC*mec* V+SCC*fus*) may enable the transfer of multi-drug resistance in a single transfer event. The use of fusidic acid is un-regulated in some countries and hence it may be used inappropriately in a community setting (for example in short or discontinuous doses). Inappropriate use of fusidic acid may therefore favour co-selection of methicillin-resistance among *S. aureus*. In addition, in this study, 14/18 (77%) of MR-CoNS were positive for *fusC* or *fusB*. This association of resistances among the resident flora may provide further opportunity for dissemination of MRSA driven by fusidic acid selective

pressure. Interestingly a positive correlation between carriage of *fusC/fusB* and phenotypic resistance was observed in only 71.9 % and 53.6 % of *S. aureus* and CoNS respectively. However, induction of gene expression with fusidic acid pre-incubation gave better correlation (82.2% and 76.3 % correlation).

There were limitations to this study, which included; a single centred, relatively small study. Some nasally abundant CoNS species, for example *Staphylococcus lugdunensis* and *Staphylococcus hominis,* were under-represented as the enrichment method favoured pathogenic staphylococci such as *S. aureus* and *S. epidermidis*. CoNS was investigated only in those co-colonised with *S. aureus* and therefore prevalence rates for genes among CoNS do not reflect the entire cohort. CCs and STs were determined only among *S. aureus* as the high rate of genetic recombination among CoNS makes strain typing unreliable. Although the microarray system used is reported effective for staphylococcal species other than *S. aureus* [35], some gene targets may be heterologous among staphylococci leading to false negatives. The study design did not facilitate follow-up of this cohort during clinical training which may have revealed further changes in gene carriage among colonising staphylococci. However, the multi-national origin of the student body in our institution facilitated analysis of a relatively broad geographic cohort in a single study and emphasises the role that importation plays in *S. aureus* epidemiology. Unlike other studies of staphylococci in the healthy human nares, pairs of staphylococcal species originating from the same individual were investigated here for their resistance and virulence traits. These data support a low rate of transfer of antibiotic resistance between colonising staphylococcal species in the absence of healthcare contact. However, it is concerning that similar SCC*mec* and SCC*fus*C types, in addition to *ileS2, qacA/qacB* and ACME are carried among CoNS and *S. aureus* in healthy individuals who will have subsequent roles in healthcare provision. Given the increasing emergence of HA-MRSA with features of community strains, further mobilisation of these elements under

selective antibiotic pressure may enhance the transmission and success of *S. aureus* in the healthcare environment.

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Competing interests

HH has received funding from Pfizer and Astellas outside the relevance of the submitted work, all other authors report no competing interests.

Contributor Statement

PEB and DFH recruited students to the study, PEB conducted the laboratory work and drafted the manuscript. DFH and HH conceived of the study and contributed to study design. AS, PK and DC provided critical data interpretation and revised the drafted work. All authors contributed to the final approved draft.

Data sharing statement

All data for these analyses are included in the manuscript. No additional data are available.

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Figure Legends

Figure 1. **Geographical origin of medical students recruited**. The geographical areas of origin of 444 medical students recruited to the study are shown (dark grey

bars). Of those recruited, 137 were confirmed nasal *S. aureus* and CoNS positive. The proportion of recruited students from each geographical origin with nasal *S. aureus* carriage are also shown (light grey bars).

Figure 2. Genotypic diversity of 137 *S. aureus* nasal isolates using DNA microarray analysis, including 128 MSSA (dark grey bars) and 9 MRSA (light grey bars). Letter (a) indicates internationally disseminated clones into which SCC*mec* can integrate. CC=clonal complex.

Table 1. Staphylococcus species recovered from 444 nasal swabs.

Staphylococcus species recovered	Total n, (%)	Methicillin-resistant phenotype n, (%)
	n=444	n=137[a]
S. aureus	137, (30.8)	
MRSA		9, (6.6)
S. epidermidis	364, (81.9)	
MRSE		17, (12.4)
S. haemolyticus	14, (3.1)	
MRSH		0
S. saprophyticus	8, (1.8)	
MRSS		1, (0.72)
Co-carriage species		
S. aureus + *S. epidermidis*	137, (30.8)	
MRSA + MR-CoNS		1, (0.72)
fusC positive *S. aureus* + CoNS		1, (0.72)

[a] CoNS were investigated only in those positive for nasal *S. aureus* in the student cohort and not in all those recruited. MRSA= methicillin resistant *S. aureus*, MRSE = methicillin resistant *S. epidermidis*, MRSH = methicillin resistant *S. haemolyticus*, MRSS = methicillin resistant *S. saprophyticus*

434 Table 2. Resistance/virulence genes detected among 137 co-located nasal *S. aureus*/CoNS pairs.

Detected Gene(s)	**Phenotypic resistance/trait**	**No. isolates positive n (%)**			**No. *S. aureus*/CoNS pairs positive (n)**
		S. aureus **n=137**	**CoNS n=137**	***P* value**	
Antibiotic resistance gene					
blaZ	β-lactam	101 (73.7)	92 (67.1)	0.289	74
fusB	Fusidic acid	2 (1.5)	27 (19.7)	0.0002*	0
fusC[a]	Fusidic acid	31 (22.6)	25 (18.2)	0.159	1
mecA	Methicillin	9 (6.5)	18 (13.1)	0.103	1
ileS2	Mupirocin	1 (0.7)	11 (8.0)	0.005*	1
*qac*A and *qac*C	Quartenary ammonium salts	3 (2.2)	29 (21.2)	<0.0001*	0
tet(K) and tet(M)	Tetracycline	13 (9.5)	6 (4.4)	0.152	0
erm(C)	Macrolide/lincosamide	6 (4.3)	5 (3.6)	1.000	0
msr(A)	Macrolide	2 (1.45)	15 (10.9)	0.002*	1
mph(C)	Macrolide	0	15 (10.9)	<0.0001*	0
dfrS1	Trimethoprim	0	19 (13.8)	<0.0001*	0

vga	Streptogramin A	1 (0.7)	6 (4.3)	0.120	0
Virulence					
ACME-*arc*	pH tolerance	1 (0.7)	44 (32.1)	<0.0001*	1
tst1[b]	Toxic shock toxin	33 (24.1)	2 (1.5)	<0.0001*	0

[a]associated with SCC element, (*ccrA-1* and *ccrB-1*) in 13/137 *S. aureus*. [b]*tst1* confirmed by PCR. ACME = Arginine Catabolite Mobile Element. * indicates a statistically significant result by Fisher's exact test.

Table 3. Fusidic acid MICs for *S. aureus* and CoNS

MIC Interpretation[a]	MIC ≤ 1 µg/ml S n (%)	MIC ≥ 1 µg/ml R n(%)	MIC ≥ 32 µg/ml HR n (%)
***S. aureus* (n = 32)**	9 (28.1)	20 (62.5)	3 (9.3)
CoNS (n = 38)	18 (47.4)	15 (39.5)	5 (13.2)

[a]Interpretation based on The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1, 2017. http://www.eucast.org, S = Susceptible, R = Resistant, HR = high level resistant

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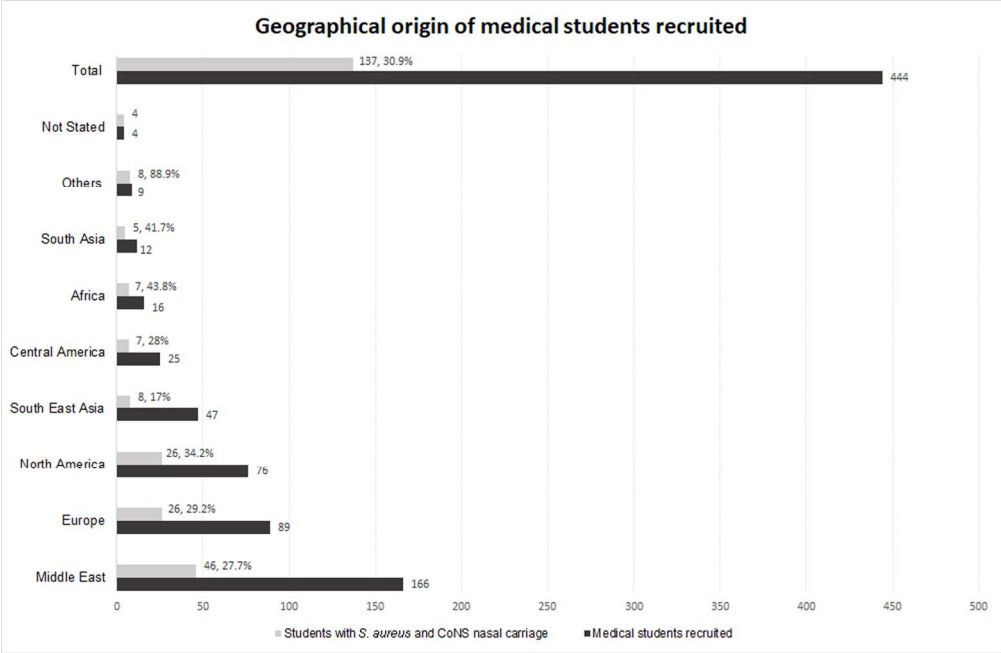

Figure 1. Geographical origin of medical students recruited. The geographical areas of origin of 444 medical students recruited to the study are shown (dark grey bars). Of those recruited, 137 were confirmed nasal *S. aureus* and CoNS positive. The proportion of recruited students from each geographical origin with nasal S. aureus carriage are also shown (light grey bars).

147x95mm (300 x 300 DPI)

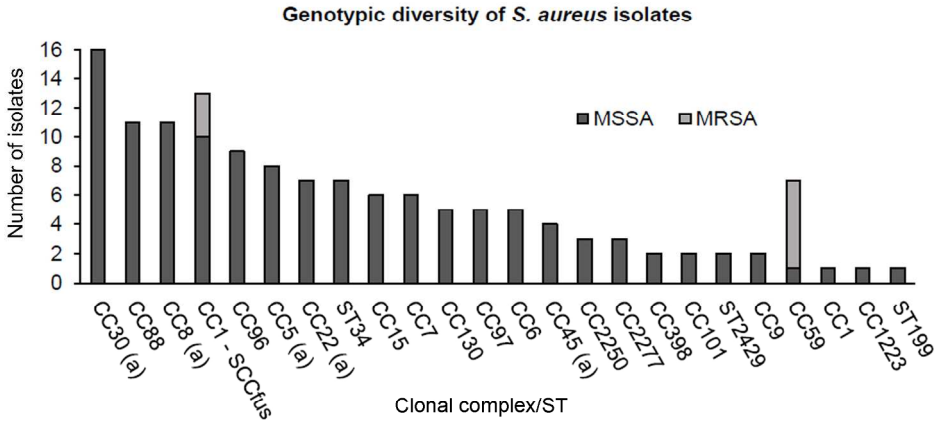

Figure 2. Genotypic diversity of 137 *S. aureus* nasal isolates using DNA microarray analysis, including 128 MSSA (dark grey bars) and 9 MRSA (light grey bars). Letter (a) indicates internationally disseminated clones into which SCC*mec* can integrate. CC=clonal complex.

222x127mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Relevent lines in manuscript and confirmation of items
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1- Title 1-Abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Yes
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Yes
Objectives	3	State specific objectives, including any prespecified hypotheses	25-29 (Abstract) 93-97 (Introduction)
Methods			
Study design	4	Present key elements of study design early in the paper	100
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	99-113
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	103-106
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	n/a n/a
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	n/a
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	134-138 (Identification of genes) 146-149 (fusidic acid susceptibility)

			110-111 (participant details, age range, region of orignin, healthcare exposure)
Bias	9	Describe any efforts to address potential sources of bias	102-106 (anonymous data collection, high participation rate no healthcare contact reported etc)
Study size	10	Explain how the study size was arrived at	105
Quantitative variables	11	Explain how quantitative variables werehandled in the analyses. If applicable, describe which groupings were chosen and why	152-154 (statistical analyses of variables)
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	152-154
		(*b*) Describe any methods used to examine subgroups and interactions	n/a
		(*c*) Explain how missing data were addressed	n/a
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	n/a n/a n/a
		(*e*) Describe any sensitivity analyses	

Continued on next page

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	105, 158
		(b) Give reasons for non-participation at each stage	n/a
		(c) Consider use of a flow diagram	n/a
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	164-165 (relevant demographic region of origin)
		(b) Indicate number of participants with missing data for each variable of interest	n/a
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	n/a
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	*n/a*
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	*n/a*
		Cross-sectional study—Report numbers of outcome events or summary measures	*158,159 S. aureus and MRSA colonisation* *162-163 MRSE among S. aureus colonised* *163-163 Co-carriage of MRSA MRSE*
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	179-183 SCCmec types among MRSA 183-186 SCCmec types among CoNS 189-194 fusC carriage among S. aureus and CoNS 200-201 Co-carriage of fus C 205-209 fusidic acid resistance among S. aureus/CoNS Confounders – n/a
		(*b*) Report category boundaries when continuous variables were categorized	n/a
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	n/a
Discussion			
Key results	18	Summarise key results with reference to study objectives	249-254 257-260 285-288 300-303
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both	330-341

		direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	YES
Generalisability	21	Discuss the generalisability (external validity) of the study results	285-290 – comparison to other studies, stability of findings in relations to carriage rates
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	356-358

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.