Original Article

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Molecular characterization of multi-drug resistant coagulase negative cocci in non-hospital environment

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Summary **Antibiotic resistance crisis occasioned by sporadic appearance of multi-drug resistance (MDR) in human pathogens to clinically applied antimicrobials is a serious threat to global health. In this study, we investigated the drug resistant phenotype of Gram-positive cocci isolates from environment. *Staphylococcus capitis* and *Staphylococcus haemolyticus* colonies were isolated on mannitol-salt agar plates supplemented with tetracycline. Antibiotic susceptibility profile of the isolates *via* minimum inhibitory concentration (MIC) determination was examined. Isolates showed decreased sensitivity to clinically applied antimicrobial agents: tetracycline, kanamycin, erythromycin, norfloxacin, teicoplanin, and ampicillin. Genomic analysis demonstrated the presence of multiple antibiotic resistant genes in these bacteria, suggesting the origin of the multiple antimicrobials resistant phenotype. Tetracycline resistance of these isolates was transduced to *Staphylococcus aureus*-RN4220 strain. These findings indicate the presence of multiple antimicrobials resistant *S. capitis* and *S. haemolyticus* strain in a non-hospital setting. Moreover, the presence of plethora of genes responsible for MDR suggest that these strains could present potential threat to human health by serving as reservoir for lateral transference of antimicrobial resistance conferring foreign genetic elements to other clinically relevant pathogens.**

Keywords: MDR, foreign genetic elements, micro-broth dilution, infectious diseases, antimicrobial resistance determinants, antibiotic resistance crisis

1. Introduction

Rampant increase in drug resistance among human pathogens has hampered antimicrobial effectiveness and limited available therapeutic options. Estimated not less than 700,000 people die annually because of antimicrobial resistant infections and is expected to rise to 10 million annual deaths by 2050 costing global economy USD100 trillion if the current trajectory is not halted (*1*). Understanding the various processes associated with drug resistance may provide important insights into new preventative and therapeutic strategies against resistant infections. Antimicrobial resistance (AMR) determinants are present as transposons, integrons or plasmids; which are utility vehicles for genetic transference to other clinically relevant microbes. AMR is complicated by the horizontal gene transfer mechanisms vis-a-vis conjugation, transduction and transformation into clinically relevant pathogens (*2-4*). Considering the foregoing, accurate identification and proper antimicrobial profiling of pathogenic species are necessary for tracking the epidemiology and treatment of infected patients in health-care settings. The environment is a reservoir of AMR genes and mobile genetic elements that are actively involved in resistant gene mobilization and transfer (*5*). Environmentally-borne dissidents are key threats to human health because of the increasing relevance of zoonotic diseases and their importance in predicting outbreak of infectious diseases (*6-9*). Despite all efforts to understand and abrogate AMR, it is evident that all is not well and further

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efforts including scientific investigations are therefore warranted. Coagulase-negative *staphylococci* (CoNS) are gradually becoming important pathogens associated with nosocomial infections. *Staphylococcus capitis*, a sub-group of CoNS and a colonizer of human intestinal tract, has been increasingly implicated in several infective diseases including endocarditis, prosthetic valve endocarditis, and late-onset sepsis in very-low birth-weight neonates. *S. capitis* has biofilm production capacity especially on medical placement devices, which contributes to its capacity to invade surgical disinfectants (*10*). *S. haemolyticus* is another pathogenic CoNS. Genomic plasticity accounting for several insertion sequences and plasmids some of which confer multiple antibiotics resistance, notably against beta-lactams and glycopeptides in these strains, has been suggested (*11,12*). As a nosocomial pathogen, *S. haemolyticus* is primarily a blood culture isolate with capacity to invade immune defenses of compromised individuals. However, data on CoNS isolation from non-hospital environment and genetic resistance mechanisms are sparsely available. In this study, we characterized the molecular mechanisms of multiple antimicrobial resistant *S. capitis* and *S. haemolyticus* strains isolated from nature and experimentally challenged the possibility of horizontal gene transmission to *S. aureus*.

2. Materials and Methods

2.1. *Isolation of strains and determination of minimum inhibitory concentration*

Colonies were isolated from the environment using mannitol-salt agar medium plates supplemented with tetracycline; 2 µg/mL. Minimum inhibitory concentration (MIC) was determined according to the following procedure. Isolated single colony was cultured overnight in tryptic soy broth medium overnight. Full growth was diluted with sterilized physiological saline to give optical density at 600 nm (Shimadzu UV-1280 spectrophotometer), 0.5 and re-diluted (1/150). Re-diluted culture (100 µL) was served with the antibiotics (100 µL) into 96-well plate, two-fold decimally diluted and incubated at 37°C. Cation-adjusted Mueller Hinton broth (CA-MHB) supplemented with calcium (25 mg/mL) and magnesium (12.5 mg/mL) ions were employed for all dilutions. MIC was defined as the lowest concentration of antimicrobial agent that inhibits growth of the organism in the micro-dilution wells as detected by the unaided eye, using the 96-well micro-titer plate format.

2.2. *Genomic DNA isolation, whole genome sequencing and assembly*

Total genomic DNA from 5 mL full growth was isolated using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following manufacturer's recommendation. The integrity and purity of the DNA was confirmed by agarose gel electrophoresis and NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Whole genome sequencing was performed using 100 ng of the total DNA as quantified by Qubit 3.0 fluorometer (ThermoFisher) as described previously (*13*). Briefly, the barcoded library of 400 base-reads was prepared using the Ion Xpress™ Plus Fragment Library Kit (ThermoFisher). The quality, quantity, and size distribution of the libraries were determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States). The libraries were then enriched in an Ion 318™ Chip v2 using Ion Chef (ThermoFisher), and subsequent sequencing was performed in the Ion PGM System (ThermoFisher). The reads were then assembled using SPAdes 3.11 (*14*) and further analysis of the assembly was performed using CLC Genomics Workbench ver 11.0 (Qiagen Bioinformatics, Aarhus, Denmark).

2.3. *Conjugation assay*

S. aureus (RN4220 strain) selected with fusidic acid (6.3 µg/mL) and rifampicin (1 µg/mL) was employed as recipients. One mL full growth (about 10^9 CFU each) of donors (*S. capitis*, *S. haemolyticus*) and recipients were mixed on ice, and centrifuged (5,000 rpm, 4°C, 5 min) and dried on 0.22-µm membrane filter (Millipore Sigma, MA-USA). Donors and recipient control experiments were also included. Conjugation was performed on Brain Heart Infusion-agar (1.5%) overnight at 37°C. Cells were recovered in sterilized saline and transconjugants were selected on BHI-agar supplemented with fusidic acid (6.3 µg/mL), rifampicin (1 µg/mL) and tetracycline (1 µg/mL). Incubation at 37°C was performed for 48 hs.

2.4. *Statistical analysis*

Appearance ratio of transconjugants was compared using Graph Pad Prism 5. $*p < 0.01$ was considered statistically significant by unpaired student's *t*-test with 99% confidence interval.

2.5. *Data availability*

This whole-genome project including the assembled contigs and raw reads has been deposited at NCBI Bio Project under the accession PRJNA471195. The DDBJ/ENA/GenBank accession numbers of each assemblies are: RIYT00000000, RIYU00000000, and RIYV00000000 for Tc^R-3, Tc^R-5, and Tc^R-7, respectively.

3. Results

3.1. *Antibiotic susceptibility profiling of isolates*

Table 1. Identification of isolates

Strain	Closest homolog	% identity, gap
Tc^R-3	*Staphylococcus capitis* strain AYP1020	1554/1554 (100%), 0
Tc^R-5	*Staphylococcus haemolyticus* JCSC1435	1554/1554 (100%), 0
Tc^R-7	*Staphylococcus haemolyticus* JCSC1435	1554/1554 (100%), 0

To characterize the isolates, 16S rDNA of whole genome analyzed isolates were extracted and blasted in National Center for Biotechnological Information (NCBI) database.

We focused on the isolation and molecular characterization of environmentally mobilized staphylococci bacteria with antibiotic resistant phenotype. Using tetracycline supplemented mannitol-salt agar medium, we isolated antibiotic resistant bacteria colonies. Cells having mannitol utilization capacity were isolated. Single colony isolation was performed using Brain Heart Infusion-agar plate. To identify the bacteria, we extracted the 16S rDNA of the sequenced isolates from the whole genome data. BLAST algorithm was performed in NCBI database. Result suggested that analyzed strains were *S. capitis* and *S. haemolyticus*, with 100% similarity to available literature data (Table 1). Antimicrobial susceptibility assay of these isolates via minimum inhibitory concentration (MIC) determination was determined. Antibiogramic data (Table 2) showed that the isolates displayed tetracycline resistance. Further examination suggested that the environmental bacteria also displayed decreased sensitivity (elevated MICs) against mechanistically diverse antimicrobial agents compared to CLSI standard breakpoints (*15*) including norfloxacin, kanamycin, and erythromycin with no observed decreased susceptibility against rifampicin, chloramphenicol and vancomycin. These findings indicate that the isolates possess genetic mechanisms that allow for its existence even in the face of environmental stress.

Table 2. Antibiogram of isolated environmental bacteria

Antibiotic	MIC (μg/mL)		
	Tc^R-3	Tc^R-5	Tc^R-7
Tetracycline	128	32	32
Kanamycin	128	1	1
Norfloxacin	1	128	128
Erythromycin	0.5	32	32
Teicoplanin	0.5	4	4
Ampicillin	1	8	8
Cefcapene	1	1	1
Cefditoren	0.5	0.5	0.5
Oxacillin	0.25	0.25	0.25
Chloramphenicol	8	4	4
Rifampicin	< 0.008	< 0.008	< 0.008
Vancomycin	2	1	1

Table 3. General feature(s) and genome statistics of the assembled sequences

Feature of chromosome	Value for strain		
	Tc^R-3	Tc^R-5	Tc^R-7
Length of sequence (bp)	2,515,208	2,563,958	2,542,447
G+C content (bp)	824,989	835,851	828,838
G+C contents (%)	32.8	32.6	32.6
Number of reads	1,476,795	603,500	643,974
Coverage	160	66	73
N50 (bp)	466,109	94,107	79,590
L50 (No. of contigs)	2	12	9

3.2. *Diversity of antimicrobial resistant genes in the isolates*

To provide genetic evidence and elucidate the molecular mechanisms behind the multidrug resistance of the isolates, we intended to identify the resistant genes in the isolates by whole genome sequencing. Genomic DNA of representative multi-drug resistant isolates (Tc^R-3, Tc^R-5 and Tc^R-7) were extracted, sequenced and assembled. Sequence data assembly revealed a genome size range of 2.5 M bp. G + C content was approximately 33%. Reads (1.48×10^6, 6.03×10^5 and 6.44×10^5) and genome coverages (160, 66, and 73) for Tc^R-3, Tc^R-5, and Tc^R-7 respectively were obtained (Table 3). Genome annotation revealed the presence of antibiotic resistance genes: *aac*(6')-*aph*(2'), *blaZ*, *mph*(C), *msr*(A), *vga*(A) and *Tet*(K) (Table 4).

3.3. *Roles of coagulate negative Staphylococci in spread of antibiotic resistance*

To understand the role of these isolates in spreading of antibiotic resistance, conjugation assay was performed using *S. aureus* RN4220, a laboratory strain, harboring fusidic acid and rifampicin resistances as recipients. Assay was performed as described in methodology section. Results revealed that the colony-forming units (CFUs) of *S. aureus* RN4220 cells that appeared on the selection plate after conjugation increased and significantly differed from each recipient controls (Figure 1). Donor controls yielded no colonies on selection plate.

4. Discussion

Notwithstanding calls for better picture on the reservoir of genes in our surrounding and how they aid acquisition of resistance phenotypes in clinically relevant pathogens; available genetic information are sparse on the resistome of environmental bacteria. In that regard, this study

Table 4. Drug resistant genes identified in the assembled genomes

Strain	Closest bacteria homolog	Identified gene	Gene function
Tc^R-3	*S. capitis*	*aac(6')-aph(2')*	Aminoglycoside resistance
		Tet (K)	Tetracycline resistance
Tc^R-5	*S. haemolyticus*	*blaZ*	Beta-lactam resistance
		mph(C)	Macrolide resistance
		msr(A)	Macrolide, Lincosamide and Streptogramin B resistance
		vga(A)	Streptogramin B resistance
		tet(K)	Tetracycline resistance
Tc^R-7	*S. haemolyticus*	*blaZ*	Beta-lactam resistance
		mph(C)	Macrolide resistance
		msr(A)	Macrolide, Lincosamide and Streptogramin B resistance
		vga(A)	Streptogramin B resistance
		tet(K)	Tetracycline resistance

The nucleotide sequences of the assembled genomes were analyzed in the CLC Genomics Workbench against the database of Find Resistance using default parameters.

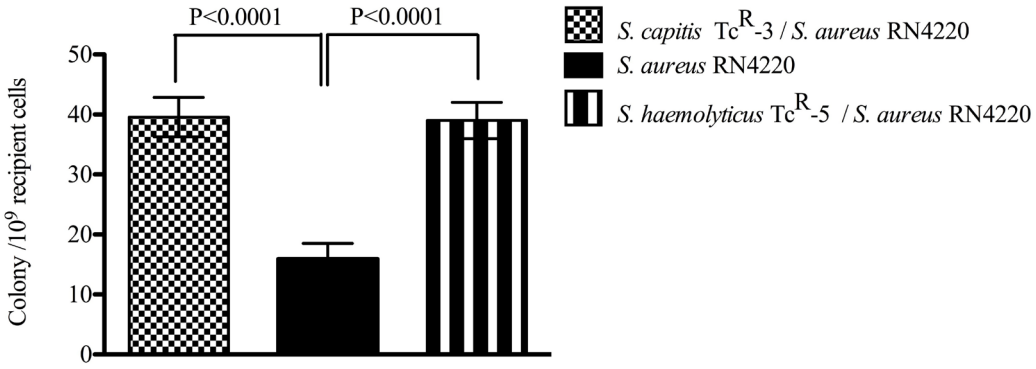

Figure 1. Conjugational transduction of tetracycline resistance to *S. aureus*. Conjugation assay was performed on BHI-agar media. To determine total number of recipient present after conjugation, recovered suspensions were diluted and spread on selective media plate and ratio of transconjugants to total recipient cells determined after 48hrs. Experiments were repeated thrice in triplicates. Plots show mean and standard deviation. Statically analysis was performed by unpaired Student's *t*-test with 99% confidence interval.

investigated multiple antimicrobials resistant bacteria isolated from a non-clinical environment in Japan. In addition to clinically relevant antimicrobial agents, these strains can transport environmental biocides and disinfectants which may allow persistence in the environment and subsequent transmission on to a suitable host. Molecular analysis of three isolates from the library showed the presence of multiple antibiotic resistant genes: *aac*(6')-*aph*(2'), *bla*Z, *mph*(C), *msr*(A), and *Tet*(K) suggetsing the origin of the MDR phenotype. Decreased sensitivity to tetracycline in staphylococci mediated by expression of a ribosomal protection protein molecule encoded by *Tet*(K) or *Tet*(M) genes, or via efflux mechanisms has been investigated (*17*). Aminoglycoside-modifying enzyme gene, *aac*(6′)-*aph*, is the most common antimicrobial resistant gene conferring aminoglycoside, including kanamycin resistance, in staphylococci. The capacities of the *blaZ* and *msr*(A) genes in conferring beta-lactams and macrolide resistances respectively are well-known. In particular, *msr*(A) gene encode ATP-dependent efflux pump conferring resistance to 14- and 15-membered macrolides, including erythromycin. Still, none of the identified genes is associated with decreased sensitivity to norfloxacin and teicoplanin. The *mph*(C) and *msr*(A) are key candidates genes, though none is previously associated with efflux of fluoroquinolones or glycopeptides. Further studies are required to elucidate the actual resistance mechanisms. This data supports the need for the controlled use of antibiotics and establishes the criticality of CoNS as a reservoir of antibiotic resistant determinants. In the knowledge of the authors, this is the first study to document the isolation of *S. capitis* harboring multiple antibiotic resistant genes from non-clinical (non-hospital) environment. Despite untiring efforts towards analyses of resistant clinical bacterial isolates, the exact process of the acquisition of resistance to these antimicrobials remains unclear. For environmental microbial resistant armamentarium, the stories are not different and further studies are thus necessitated. Understanding these mechanisms could potentially aid antimicrobial agents development. Presence of plethora of genes responsible for antibiotic resistance suggests

that strains *S. haemolyticus* and *S. capitis* could present a potential threat to human health. Environmentally mobilized MDR bacteria such *S. haemolyticus* and *S. capitis* might serve as a foothold towards transference of AMR conferring foreign genetic elements into other clinically relevant pathogens.

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Conflict of Interest

K.S. is a consultant for Genome Pharmaceuticals Institute Co., Ltd.

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