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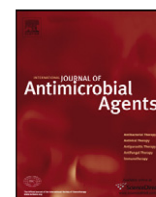

Expression of multidrug resistance efflux pump genes in clinical and environmental isolates of *Staphylococcus aureus*

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ABSTRACT

Increased expression of multidrug resistance efflux pump (MDR-EP) genes in clinical isolates of *Staphylococcus aureus* occurs frequently, but its temporal and geographic variability is unknown. Such strains may contaminate the hospital environment, posing an infection control problem. Nearly 700 clinical isolates from different geographic locales as well as 91 environmental isolates recovered from two Detroit hospitals were studied. Ethidium bromide (EtBr) minimum inhibitory concentration (MIC), quantitative expression of all characterised chromosomal MDR-EP genes, and the presence of *qacA*/*B* and *smr* were determined for all strains. In addition, for *norA*- and/or *mepA*-overexpressing strains, the *spa* type was established. MDR-EP gene overexpression varied temporally and geographically, and overexpressing strains were present in the hospital environment. Increased expression of *norA* was associated with meticillin resistance and *spa* type t002, a rare type among control strains, consistent with widespread dissemination of a *norA*-overexpressing, meticillin-resistant *S. aureus* (MRSA) clone. Clonal spread also played a role for *spa* type t008, *mepA*-overexpressing, meticillin-susceptible strains. An EtBr MIC of ≤12.5 μg/mL was highly specific (>90%) in identifying strains lacking MDR-EP gene overexpression.

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1. Introduction

Bacterial multidrug resistance limits therapeutic options. This phenotype results from the concurrence of several resistance mechanisms, including drug inactivation, target-based mutations compromising drug binding, reduced drug permeability and thus target access, and membrane-based efflux proteins. Some efflux proteins have broad substrate profiles and are referred to as multidrug resistance efflux pumps (MDR-EPs) and are found both in Gram-positive and Gram-negative bacteria [1]. Genome sequence data for *Staphylococcus aureus* N315 have identified at least 30 genes encoding putative drug transporters, most of which are major facilitator superfamily (MFS) proteins [2]. Many of these MDR-EPs may have natural functions, with drug efflux being a fortuitous event [3].

We previously established that increased expression of MDR-EP genes is common among bloodstream isolates of *S. aureus* recovered from inpatients at Detroit Medical Center hospitals (Detroit, MI) [4,5]. We also showed that augmented expression was present in a small number of geographically and temporally diverse clinical *S. aureus* strains, indicating that this phenotype has existed for decades [6]. Whether or not these data can be generalised to contemporary strains from various geographic locales is unknown.

Staphylococcus aureus can contaminate the hospital environment and in so doing may represent an infection control hazard [7,8]. Quaternary ammonium biocides are frequently employed for routine hospital disinfection, and these compounds are substrates for most *S. aureus* MDR-EPs [2]. Efflux-proficient mutants can emerge following in vitro exposure to quaternary ammonium compounds, and it is possible that the same may be true for environmental strains [9]. The emergence and subsequent survival of MDR-EP-overexpressing strains may be favoured by a protected environmental niche and/or organic debris, which then may be transmitted to patients and subsequently cause infection [10]. Whilst it is likely that efflux-proficient strains do exist in the hospital environment, data supporting their presence are lacking.

In this study, several hundred unique clinical isolates of *S. aureus* from hospitals in five geographically distinct US cities as well as one in Freiberg, Germany were collected. The environment of two Detroit hospitals was also cultured and clinical and environmental isolates from these institutions were compared. Strains overexpressing MDR-EP genes were found to be common, widely distributed geographically, mainly meticillin-resistant, clonally

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related with respect to *norA* and *mepA* overexpression, and present in the hospital environment.

2. Materials and methods

2.1. Bacterial strains, media and reagents

One bloodstream isolate of *S. aureus* per patient as well as nafcillin susceptibility data were collected during 2009 from the microbiology laboratories of hospitals in the Detroit Medical Center, Boston, MA, Houston, TX, Omaha, NE, and Freiberg, Germany ($N = 563$). An additional 126 unique non-bloodstream isolates were obtained from centres in San Francisco, CA. Environmental isolates from two Detroit hospitals were obtained by the methods described below ($N = 91$). *Staphylococcus aureus* SH1000 was used as the control strain for quantitative real-time RT-PCR (qRT-PCR) [11]. Unless otherwise noted, all reagents were the highest grade available and were obtained from Sigma Chemical Co. (St Louis, MO) or BD Biosciences (Sparks, MD).

2.2. Antimicrobial susceptibility testing

Previous work using a subset of strains included herein revealed that the ethidium bromide (EtBr) minimum inhibitory concentration (MIC) had excellent sensitivity and specificity (95% and 99%, respectively) in identifying strains possessing an efflux phenotype as well as good specificity (92%) in identifying strains lacking overexpression of MDR-EP genes [12]. As such, the EtBr MIC can be used as a surrogate marker for identifying strains of these types. EtBr MICs were determined in triplicate for all strains by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. MICs of benzalkonium chloride (BAC), selected as a representative quaternary ammonium biocide, were also determined for 40% of the study strains.

2.3. qRT-PCR

Seven *S. aureus* chromosomal genes are known to encode MDR-EPs, including *mdeA*, *mepA*, *norABC*, *sdrM* and *sepA* [14–20]. All of these except *mepA* and *sepA* encode 12 transmembrane segment (TMS) MFS proteins. qRT-PCR was performed using a multiplex approach as described previously with a QuantiTect® Multiplex RT-PCR Kit (QIAGEN, Inc., Valencia, CA) and an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) [12]. Beacon Designer 7.80 (Premier Biosoft International, Palo Alto, CA) was used to design TaqMan® probes and primers, which were purchased commercially (Eurofins MWG Operon, Huntsville, AL). Positive controls for each gene were included, and 16S cDNA was used as the endogenous control. The presence of *qacA* or *qacB*, which are plasmid-based genes encoding highly related MDR-EPs, was detected by PCR using primers designed to amplify a homologous region of both genes. The presence of plasmid-based *smr*, also known as *qacC* and encoding a 4 TMS small multidrug resistance (SMR) family protein, was detected by PCR using published primers [21]. The comparative threshold cycle (C_T) method was used to determine relative gene expression compared with that of *S. aureus* SH1000, in which expression of each gene was considered to be 1.0. Values of ≥4.0 were considered indicative of gene overexpression.

2.4. Environmental culturing

Environmental isolates of *S. aureus* were recovered from patient care areas in two Detroit hospitals using Rodac contact plates (25 cm^2 surface area; BD Biosciences) containing mannitol salt agar (MSA) (Remel, Lenexa, KS). Both hospitals employed quaternary ammonium biocide formulations for environmental disinfection, and cultures were obtained prior to daily room cleaning. Eighteen surfaces in patient rooms were sampled including medical, surgical, intensive and long-term care settings. Cultured surfaces included floors, bedside tables, bed rails, door and sink handles. Surface conformations without an appropriately sized flat component were sampled using a 25 cm^2 template and a cotton-tipped applicator moistened with brain–heart infusion broth (BD Biosciences), which was then streaked onto an MSA plate. Plates were incubated for 24 h at 35 °C. Colonies producing a yellow discoloration of the medium were identified presumptively as *S. aureus* and were confirmed as *S. aureus* by coagulase testing. One coagulase-positive isolate per culture location, if available, was selected for further analysis.

2.5. Strain typing

The observed preponderance of meticillin-resistant *S. aureus* (MRSA) and meticillin-susceptible *S. aureus* (MSSA) among strains overexpressing *norA* or *mepA*, respectively, was evaluated using *spa* typing as previously described [22]. For control purposes, *spa* types were also determined for 80 and 46 non-MDR-EP gene-overexpressing MRSA and MSSA strains, respectively. Nucleotide sequences were determined using an automated dideoxy chain-termination method by the Applied Genomics Technology Center, Wayne State University (Detroit, MI) [23]. Assignment of *spa* type was made employing a web-based application at http://fortinbras.us/cgi-bin/spaTyper/spaTyper.pl, which utilises the Ridom numbering scheme as described at http://spa.ridom.de/index.shtml.

2.6. Data analyses

The utility of the EtBr MIC in predicting efflux proficiency and the absence of MDR-EP gene overexpression has already been mentioned [12]. Using the current data set, which includes twice the number of strains as reported in an earlier study as well as PCR-based detection of an additional MDR-EP gene (*smr*), the sensitivity, specificity, and positive and negative predictive values of an EtBr MIC ≥ 25 μg/mL being indicative of MDR-EP gene overexpression were determined using algorithms available at http://www.neoweb.org.uk/Additions/predict.htm. True positives were strains having MDR-EP gene overexpression and an EtBr MIC ≥ 25 μg/mL, true negatives had neither of these characteristics, false positives had an EtBr MIC ≥ 25 μg/mL without MDR-EP gene overexpression, and false negatives had MDR-EP gene overexpression and an EtBr MIC ≤ 12.5 μg/mL. Statistical analyses were performed using the *z*-test with Yates correction (SigmaPlot 12.0; Systat Software, Inc., Chicago, IL).

3. Results

3.1. Gene expression

Gene expression data and the percentage of strains from each locale that were MRSA are provided in Table 1. Data from our previous study describing *S. aureus* bloodstream isolates collected in 2005 are included for comparative purposes [5]. Aggregate data for all 2009 strains revealed that MRSA predominated among those overexpressing at least one MDR-EP gene (74%) compared with a significantly lower proportion among non-overexpressing strains (44%) ($P < 0.001$) (data not shown). This association also was observed in our earlier study [4,5].

A comparison of Detroit strains from 2005 to 2009 demonstrated that the proportion overexpressing at least one MDR-EP gene fell significantly in 2009 (36.7% vs. 55.2%; $P < 0.001$). Variations in increased expression of individual genes among Detroit isolates

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