An Improved Method for Assessing the Biocidal Activity of Antimicrobial Silver Wound Dressings Against *Staphylococcus aureus*

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Abstract

Bacterial wound infections are a concerning and persistent healthcare issue, costing an estimated 50 billion USD annually. To meet the need for better wound dressing products, more effective evaluation methods are necessary. In this study, the AATCC-100 protocol was extended in the evaluation period and illustrative of labelling. The metal-bearing dressings were less biocidal following 24 hours of use, with approximately 1 log reduction after 7 days. These data and others suggest the need for a PCR based assay to evaluate adaptive viability in response to silver exposure as well as for identifying viable but non-culturable cells (VBNCs).

Materials and Methods

Inoculation, Extraction, Harvesting:

Sterile 8 oz Nalgene Jar
Calcium Alginate Wound Dressings
D/E Neutralization buffer
Tryptic soy agar and tryptic soy broth
Bolton Broth
Fetal Bovine Serum
Staphylococcus aureus ATCC 3556
BSL 2 Safety Cabinet
37˚C Incubator and Incubator-shaker
Spectrophotometer
Liquid Nitrogen

Exponential phase *Staphylococcus aureus* (OD600~0.8), are inoculated onto wound dressings, with and without antimicrobial silver (1). A sterile control is included. The wound dressings are contained within 8 oz Nalgene bottles and are hydrated with simulated wound fluid (SWF), consisting of equal volumes bolton broth and fetal bovine serum (2). Following 1, 3, and 7 days incubation at 37˚C, the cells are extracted by vortexing in D/E neutralization buffer (3). Serial dilutions are counted from TSA plates to determine the log reduction in cells. For each timepoint extracts were also flash frozen in liquid nitrogen. Log reduction is calculated using starting inoculate in the numerator.

RNA Extraction and Reverse Transcription:

RNAeasy minikit by QIAGEN
QIAcube by QIAGEN
Nanodrop
Superscript III First Strand Synthesis System by Thermofisher

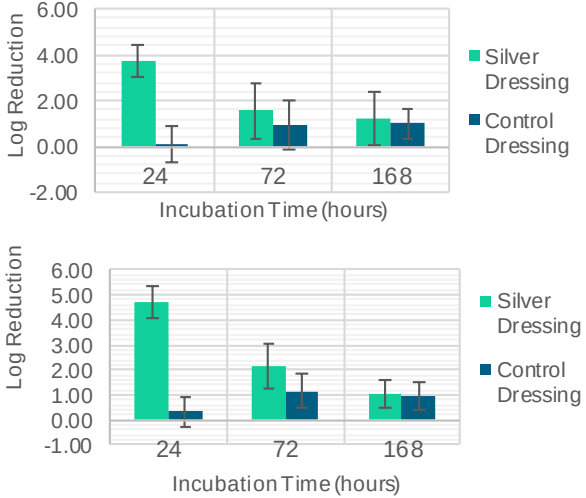
RNAeasy kit (**1**) adapted for QIAcube (**2**) disrupts thawed cell extracts in RLT, washes onto silicon membrane, and elutes purified total RNA. cDNA is generated using Superscript III kit. (**3**), which employs non-specific reverse transcription. Purity and yield is determined with Nanodrop spectrophotometer (A 260/280). This process is repeated to generate cDNA from extracts collected on days 1,3, and 7.

Quantitative RT-PCR:

UCP Probe PCR Kit by QIAGEN
BioRad real-time thermo-cycler
Housekeeping gene primer (mecA)
Genes of interest primers (hlgB,hlgC,luk-F,spa,hlb)

Primers and template are added to master mix according to UCP Probe PCR kit protocol (**1**). An initial heat activation step is followed by 40 cycles of denaturation, annealing, and elongation, mediated by BioRad thermocycler (**2**). For absolute quantification, the primer is for the housekeeping gene mecA and the template DNA consists of serial dilutions of cDNA. A standard curve based on Ct values is then generated. For relative quantification, the primers are for genes of interest associated with virulence. These genes are luk-F, hlgB, hlgC, spa, and hlb. Their expression is quantified using ΔΔCt, with the housekeeping gene as a reference.

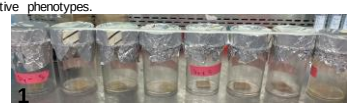
Results



S. aureus viability: replicate assays (with technical triplicates), the log reduction for the silver-bearing wound dressings decreases after 24 hours compared with control-dressings whose log reduction increases in the post 24- hour tests.

Discussion

The decrease in log reduction of the silver-bearing wound dressings is consistent with persistence and growth of *Staphylococcus aureus* in the post 24-hour period (counts not shown). Persistence may be mediated by adaptive viability. The growth of biofilms induced by biocide exposure is one potential adaptation, with biofilm cells exhibiting distinct proteomes from their planktonic counterparts [1]. Furthermore, the increased log reduction in the control dressings suggests reduced persistence phenotypes due to the absence of silver. The selection for resistant phenotypes due to biocide exposure has been well documented in the literature [2,3,4]. Interestingly, a darkish coloration was observed in the silver-exposed group that was not present in the control, possibly indicating silver-induced metabolic changes (**1**). Looking forward, RT-PCR will enable testing for cells that are not culturable (VBNC), and relative quantification will help evaluate adaptive viability in the post 24-hour period. This assay may be more effective than traditional culturing methods for assessing wound dressing antimicrobial efficacy and their effects on adaptive phenotypes.



Changes in dressing pigmentation: darker coloration distinguishable on silver dressings (rightmost 3 jars) with *S. aureus* growth

Conclusion

The wound dressings in this study were no longer biocidal after 24 hours. Culturing techniques alone do not suffice for assessing a dressing antimicrobial efficacy, resulting from inconsistencies in the implementation of this method (significant variation in plate-counting results). For these reasons, a PCR based assay may be an appropriate alternative for a more exhaustive and time-efficient protocol for evaluating wound dressings' antimicrobial efficacy.

Citations

1. Resch, Alexandra. "Differential Gene Expression Profiling of Staphylococcus Aureus Cultivated under Biofilm and Planktonic Conditions." *American Society for Microbiology* (2005).
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3. Huet, Aurelie. "Multidrug Efflux Pump Overexpression in Staphylococcus Aureus after Single and Multiple in Vitro Exposures to Biocides and Dyes." *Microbiology Society* (2008).
4. Pasquaroli, S. . "Antibiotic Pressure Can Induce the Viable but Non-Culturable State in Staphylococcus Aureus Growing in Biofilms." *Journal of Antimicrobial Chemotherapy* (2013).

Acknowledgments

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