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Major Article

Long-term metabolic persistence of gram-positive bacteria on health care-relevant plastic

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Background: Health care-associated opportunistic pathogens *Staphylococcus aureus* and *Enterococcus faecium* persist on dry environments and can contribute to organism transmission through contact. These organisms can be monitored on surfaces by culture, molecular methods, or metabolic assays. This study was designed to determine the kinetics of bacterial persistence on acrylonitrile butadiene styrene, a plastic commonly used in the manufacture of bedrails.

Materials and methods: Polymerase chain reaction for genomic DNA was used to confirm the presence of bacteria cells on this plastic irrespective of viability. Bacterial viability was measured by culture, ATP quantification, and a metabolic assay at time points up to and longer than 1 year.

Results: Polymerase chain reaction confirmed the presence of bacteria on the plastic for the entire time period studied. However, *S aureus* culturability was reduced after 3 and 7 weeks; neither organism was culturable after 1 year. At 7 weeks, ATP levels were reduced for both organisms, paralleling *S aureus* culturability but indicating that ATP quantification did not predict *E faecium* culturability. *S aureus*-reducing potential was reduced after 7 weeks, whereas *E faecium*-reducing potential reached the level of fresh inoculum after 12 hours in broth culture. Low but significant levels of metabolic activity were detected for each organism after 1 year.

Conclusions: *S aureus* and *E faecium* cells may retain viability on plastic for longer than 1 year.

Nosocomial pathogens are known to persist on surfaces in health care environments.[1-4] These organisms may be present due to fresh inoculation, after inappropriate cleaning, or to settling of cells suspended in the atmosphere. Culture is the primary method used to monitor the presence of these organisms on surfaces. In the absence of culturability, bacterial presence can also be quantified using molecular methods,[3] ATP detection,[5] and other nonculture methods such as metabolic assays.[6,7]

The gram-positive organisms *Staphylococcus aureus* and *Enterococcus faecium* can be drug resistant and pose a worldwide threat to human health.[8] These bacteria persist on dry environments such as hospital bedrails,[9] which are commonly composed of the plastic acrylonitrile butadiene styrene (ABS). This surface can act as a reservoir, contributing to the contact contamination of hands or medical equipment and leading to transmission of these organisms. The purpose of this study was to determine the kinetics of bacterial persistence on ABS. Plastic discs were inoculated with these organisms and allowed to remain at room temperature in a dry environment for periods up to and exceeding 1 year. Polymerase chain reaction (PCR) assays for genomic DNA were used to confirm the presence of bacteria cells on this surface irrespective of viability. Bacterial viability was measured by culture, ATP quantification, and a metabolic assay.

MATERIALS AND METHODS

Inoculation of plastic surfaces

We used 6-mm, 0.062-in thick ABS discs (Industrial Plastic Supply, Inc, Anaheim, CA) that were incubated in 70% ethanol for 15 minutes to minimize contaminating organisms and air-dried in a hood before inoculation. Discs were inoculated with 10 µL washed bacterial cultures of *E faecium* (ATCC 19434, Manassas, VA) or *S aureus* (ATCC 25923) suspended at 10^9 cells/mL in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD). Broth was used to mimic the proteins present in patient fluids that are potential sources of surface contamination. After drying, the plastic discs were incubated in a

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sterile petri dish at room temperature for 16 hours, 3 weeks, 7 weeks, or approximately one year.

Determination of colony forming units and PCR detection of S aureus *and* E faecium *genomic DNA*

At specific time points after incubation, the discs were suspended in sterile phosphate buffered saline and vortexed for 10 minutes to aid in cell removal. After centrifugation at 13,000 rpm for 5 minutes, the bacterial pellet was resuspended in molecular-grade water. For culture quantification, serial dilutions were spread on BHI agar, incubated 16 hours at 37°C, and colonies were quantified. Genomic DNA was extracted (High Pure PCR Template kit, Roche Diagnostics, Mannheim, Germany) and the absolute number of cells quantified by comparison to a 4-point standard curve. For standards, the concentration of purified bacterial DNA was converted to copy number based on the genomic sizes of the *E faecium* reference sequence (GenBank CP003583.1, 26,981,37 base pairs) and the *S aureus* ATCC 25923 strain (GenBank CP009361.1, 2,778,854 base pairs). The single-copy *S aureus* factor essential for expression of methicillin resistance (*femA*) gene was detected using primers femASa-F (5'-TGCCTTTACAGATAGCATGCCA-3') and femASa-R (5'-AGTAAGTAAGCAAGCTGCAATGACC-3'),[10] whereas the single-copy *E faecium* D-alanyl-alanine synthetase A (*ddl*) gene was detected using primers Ef2ddl-F (5-TTTACAAGCTGCTGGTGTGC-3') and Ef2ddl-R (5'-AACCCATATTCGCAGGTTTG-3').[11] The amplification efficiency, $E = (10^{-1/\text{Slope}} - 1) \times 100$, for each of these established primer sets was 97.6% and 92.8%, respectively. No primer dimers were detected up to 40 cycles. Amplifications were performed per the manufacturer's instructions using 2X Select SYBR Master Mix (Applied Biosystems, Austin, TX). Each 25-µL reaction contained 2×10^{-7} M forward and reverse primers, and 5 µL sample or molecular-grade water as a negative control. The generation of target for each sample was monitored during elongation on a CXF96 Thermal Cycler (BioRad, Hercules, CA).

ATP determination

The wells of an opaque 96-well plate (Corning Incorporated, Corning, NY) were inoculated with 10 µL washed bacterial cultures suspended at 10^9 cells/mL in BHI broth. After drying, the plates were covered and incubated at room temperature for 16 hours or 7 weeks. ATP detection was performed per the manufacturer's instructions (BacTiter-Glo Microbial Cell Viability Assay, Promega Corporation, Madison, WI) and luminescence quantified (Fluostar Omega, BMG Labtech, Cary, NC).

Metabolism by reducing ability

At specific time points, the discs were transferred to the wells of a 48-well plate and BHI broth containing PrestoBlue (Invitrogen, ThermoFisher Scientific, Irvine, CA). This reagent contains cell permeable resazurin, which is reduced to a fluorescent compound by bacterial enzymes, primarily reductases and dehydrogenases. Plates were incubated 16 hours with hourly monitoring (excitation, 544 nm; emission, 620 nm; Fluostar Omega) and compared with uninoculated control discs.

Statistical analysis

The statistical significance between the groups was determined by 1-way analysis of variance with Tukey-Kramer multiple comparisons test (GraphPad Software, La Jolla, CA). Student *t* test was used to compare metabolism at 1 year to background. A *P* value < .05 was considered significant.

RESULTS

A PCR assay detecting the genome of each organism was used to quantify total inoculated bacteria present on the ABS surface irrespective of viability. This detection method is initially compared with the culturability of each organism after removal by swabbing. No variation in *S aureus* number as detected by PCR was noted at 1 year (Fig 1A). However, *S aureus* culturability was reduced by 2.5 Log_{10} ($P < .001$) after 3 weeks, by an additional 2 Log_{10} ($P < .001$) after 7 weeks, and was undetected after 1 year. This indicated that although the bacteria are still present on the surface, they are not viable as indicated by culture. The *E faecium* colony forming unit count at 16 hours and 3 weeks was slightly although significantly greater than the genomic DNA quantification by PCR ($P < .01$). The colony forming unit count marginally decreased ($P < .001$) after 7 weeks on plastic and cells were not culturable after 1 year (Fig 1B).

Assays determining cellular ATP levels are commonly used in health care facilities for quality control to detect residual bacterial contamination on surfaces. In this study, ATP assays were performed immediately and after 7 weeks' incubation on plastic to parallel the culture and PCR results. After 7 weeks, only low levels of ATP were detected for both *S aureus* and *E faecium* (Fig 2). This assay supports the *S aureus* culture data, but contradicts the *E faecium* culture results.

The ability to maintain a reducing environment is an indicator of a cell's viability. A growth time course in the presence of

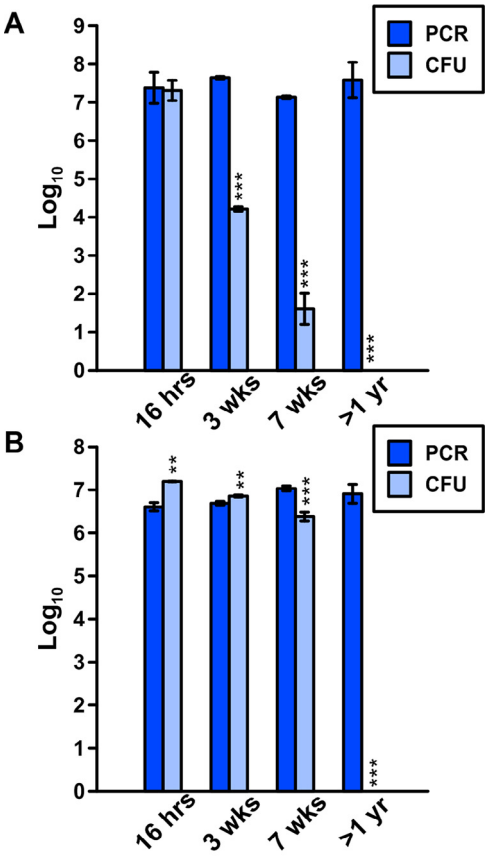

Fig 1. Culturability and presence of genomic DNA after 16 hours, 3 weeks, 7 weeks, or >1 year (*Staphylococcus aureus*, 751 days; *Enterococcus faecium*, 391 days) on acrylonitrile butadiene styrene plastic at room temperature. Whereas polymerase chain reaction detection of genomic DNA remains stable over 1 year, culturability decreases significantly, and is organism specific. (A) *S aureus*. (B) *E faecium*. N = 3. Values are presented as mean ± SD. $^{**}P < .01$; $^{***}P < .001$.