

Research Article

**Viable bacteria persist on antibiotic spacers following two-stage revision for periprosthetic joint infection<sup>†</sup>**

**Running title:** Bacteria rRNA remain on PMMA spacers

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<sup>†</sup>This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jor.23611]

**Received 26 January 2017; Revised 28 March 2017; Accepted 11 May 2017**

**Journal of Orthopaedic Research**

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**DOI 10.1002/jor.23611**

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Accepted Article

Author Contributions Statement: All authors contributed to study design, data analysis, and manuscript preparation. DM, RMS, TKW, KLU contributed to sample analysis. CMD, DWC, BRH, and KLU contributed to sample collection and preparation. All authors read and approved a final draft of the manuscript.

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## Abstract

Treatment in periprosthetic joint infection (PJI) remains challenging. The failure rate of two-stage revision and irrigation and debridement with component retention in PJI suggests that biofilm cells have a high tolerance to antibiotic chemotherapy. Previous work has demonstrated that biofilm cells have high antibiotic tolerance *in vitro*, but there is little clinical evidence to support these observations. The aim of this study was to determine if retrieved antibiotic spacers from two-stage revision total knee arthroplasty for PJI have evidence of remaining viable bacteria. Antibiotic poly (methyl methacrylate) (PMMA) spacers from two-stage revision total knee arthroplasty for PJI were prospectively collected and analyzed for bacterial 16s rRNA using polymerase chain reaction (PCR), reverse transcription (RT)-PCR, quantitative RT-PCR (qRT-PCR), and single genome analysis (SGA). PCR and RT-PCR identified bacterial species on 53.8% (7/13) of these samples. When initial culture negative cases are excluded, 68% (6/9) samples were identified with bacterial species. A more rigorous qRT-PCR analysis showed a strong positive signal for bacterial contamination in 30.7% (4/13) of cases. These patients did not show any clinical evidence of PJI recurrence after 15 months of follow-up. Because the half-life of bacterial rRNA is approximately a few days, the identification of bacteria rRNA on antibiotic PMMA spacers suggests that viable bacteria were present after conclusion of antibiotic therapy. This study provides evidence for the high tolerance of biofilm cells to antibiotics *in vivo* and the important role of bacterial persisters in PJI. This article is protected by copyright. All rights reserved

**Keywords:** Periprosthetic Joint Infection; Biofilm Antibiotic Tolerance; Bacterial Persisters; Total Knee Arthroplasty; 16S rRNA; PCR

## Introduction

Treatment in periprosthetic joint infection (PJI) remains challenging. The failure rate of two-stage revision and irrigation and debridement with component retention in PJI suggests that biofilm cells have a high tolerance to antibiotic chemotherapy<sup>1-12</sup>. There are multiple *in vitro* examples to support this. For example, after exposure to 100 times the minimal inhibitory concentration (MIC) of cefazolin, approximately 10% of a mature methicillin sensitive *Staphylococcus aureus* biofilm remain on arthroplasty materials<sup>13</sup>. Also, antibiotic loaded poly methyl-methacrylate (PMMA) bone cement can serve as a substrate for biofilm<sup>14; 15</sup>. There are many hypothesis to explain these observations including limited diffusion of antibiotics, altered metabolism, and bacterial persisters<sup>16; 17</sup>. Bacteria persisters are defined as a subpopulation of bacteria that have a phenotypically high tolerance to antibiotics. However, there is little data to support these observations in a clinical setting.

Chronic PJI and treatment with two-stage exchange affords an opportunity to test this observation *in vivo*. In chronic PJI, a two-stage revision is performed where implants are removed and replaced by an antibiotic PMMA spacer while the infection resolves. After the infection has been controlled, the antibiotic spacer is removed, and final components are then inserted. If biofilm cells have a high tolerance to antibiotics, then viable bacteria could persist on the removed antibiotic PMMA spacer, even in clinical cases where the patient has no recurrence of infection.

We hypothesized that ribosomal nucleic acid (rRNA) could be used to determine the presence of viable bacteria on removed antibiotic spacers after a two-stage exchange. rRNA has an approximate half-life of several days<sup>18; 19</sup>. The presence of rRNA six weeks after the completion of antibiotics would suggest a viable biofilm remained on the PMMA spacer in that time period. A prospective clinical study was performed to test this hypothesis. Patients undergoing a two-stage exchange for chronic TKA PJI were enrolled, and we tested for the possible presence of bacterial rRNA using multiple reverse transcriptase polymerase chain reaction (RT-PCR) techniques.

## **Methods**

### **Study Design**

A prospective, multicenter analytic cohort study of patients undergoing explant of antibiotic PMMA spacers for TKA PJI was performed (Level II). Institutional review board approval was obtained. Patients undergoing a two-stage exchange for TKA PJI with an antibiotic cement spacer were enrolled at the time of the removal of the antibiotic cement spacer. Antibiotic spacers were both articulating and static. Inclusion criteria included that antibiotic spacers did not culture any organisms after removal and that there was no evidence of infection recurrence for at least one year following the two-stage exchange. At the time of removal, antibiotic spacers were divided using an osteotome so that one part could be used for microbial culture and one part for rRNA analysis. As a control, a series of antibiotic PMMA spacers were formed in the operating room under sterile conditions, handled in a similar manner as the surgical specimens, and underwent identical handling in sample preparation.

### **Sample Preparation**

A portion of the collected antibiotic PMMA spacer was sent for microbiological culture using the institution's clinical microbiological laboratory following standard clinical protocols. The remaining spacer was then sent for analysis for bacteria rRNA. The sample was submerged in Ambion™ RNAlater® Stabilization Solution (Thermo Fisher Scientific, Carlsbad, CA). Time between specimen collection and contact with RNAlater was limited to no more than two hours. Samples were stored at -80°C.

### **Bacterial RNA and DNA isolation**

PMMA spacer samples were sonicated for 20 minutes (Branson 3510-DTH Ultrasonic Cleaner) and solid material was removed. Samples were then centrifuged at  $8,000 \times g$  for 5 minutes at 4°C. Total RNA was isolated via Ambion™ TRIzol® Max Bacterial RNA Isolation kit (Thermo Fisher Scientific, Carlsbad, CA). Invitrogen™ TURBO DNA-free™ kit (Thermo Fisher Scientific, Carlsbad, CA) was used to separate DNA contamination from total RNA. Total DNA was isolated from the interphase and phenol-chloroform layer saved from the phase separation step above according to manufacturer's recommendations.

### **Nested PCR and DNA gel extraction**

Universal oligonucleotide primers, used in bacteria 16S rRNA gene amplification, are listed in Table 1. Nested PCR is a modification of PCR intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites<sup>20</sup>. Nested PCR reactions

were performed using Invitrogen™ AccuPrime™ Pfx DNA polymerase (Thermo Fisher Scientific, Carlsbad, CA). For the first round PCR, primers utilized for the 16S rRNA gene amplification were 16S-rRNA-27-F (5'-AGAGTTTGATYMTGGCTCAG-3') and 16S-rRNA-1492-R (5'-CGGYTACCTTGTTACGACTT-3'). For the second round PCR, different sets of primers listed in Table 1 were used. Nested PCR reaction mixture was run via 1% agarose gel electrophoresis. DNA bands were extracted via QIAquick® Gel Extraction kit (Qiagen, Hilden, Germany). Oligonucleotide primers were synthesized by the Invitrogen Company (Thermo Fisher Scientific, Carlsbad, CA).

### **Single Genome Amplification (SGA) of bacterial 16S rRNA gene**

Single-cell genomics (SGA)<sup>21; 22</sup> was utilized to amplify the 16S rRNA gene from different pathogen strains from spacers with potentially polymicrobial infections. Nested PCR was performed as above by diluting the cDNA or genome DNA in the first round PCR to get less than a 25% positive rate in the second round of PCR. Then, the amplicons were subjected to agarose gel electrophoresis and gel extraction for sequencing. The PCR products were sequenced by the University of Pittsburgh HSCRF Genomics Research Core Sanger Sequencing service. Individual sequence fragments for each amplicon were inspected and edited using the BioEdit (v7.2.5) software, and followed by running BLAST to calculate sequence similarity with NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the bacteria with the highest probability of alignment to the isolated sequence from the PMMA spacer.

### **Colony formation assay**

The soft agar colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony<sup>16; 23</sup>. Titanium rods (10 mm) were incubated in TSB growth medium inoculated with  $1 \times 10^6$  CFU/ml *S. aureus* (SH1000) for 48 hours. Rods were then washed once with 1ml PBS and given fresh medium containing 10  $\mu$ g/mL cefazolin for a 28 day period. At each corresponding time point, samples were sonicated for 20 minutes and centrifuged at 8000 rpm for 5 minutes. The bacterial concentration (CFU/mL) was then determined via CFU assay on TSA II plates (Thermo Fisher Scientific, Carlsbad, CA).

### **16S rRNA Degradation assay**

Titanium rods were incubated in TSB growth medium inoculated with  $1 \times 10^6$  CFU/ml *S. aureus* (SH1000) for 48 hours. Rods were sonicated for 20 minutes and centrifuged at 8000 rpm for 5 minutes. The pellet was resuspended TE buffer (pH 8.0) and heat shock for 10 minutes at 85°C. After cool to room temperature, 10 mg/ml lysozyme and 10 mg/ml RNase A were added into it and incubated for 30 minutes at 37°C. Then, 100  $\mu$ g/ml proteinase K /0.2% SDS was added and incubated at 50°C for 10 minutes. Samples were stored at 37°C until each time point. Total RNA extraction was performed as described above on each time point over a twenty-eight-day period. The rRNA quantity was detected by standard curve qRT-PCR. Pure water was used as a negative control, and all samples were analyzed without the addition of reverse transcriptase in the reaction mixture to ensure the complete removal of DNA during RNA isolation.

### **cDNA synthesis**

Reverse transcription of RNA to single-stranded cDNA was performed using Invitrogen™ SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific, Carlsbad, CA) according to manufacturer's recommendations. The newly synthesized cDNA was used immediately or frozen at -80°C.

### **Preparation RNA Standards for Staphylococci 16S rRNA gene**

The standard curve method was selected to quantify rRNA copies on the PMMA spacer, as it is a simple and reliable assay that avoids the practical and theoretical problems associated with PCR efficiency assessment<sup>24</sup>. Possession of a good standard RNA template is a prerequisite for this method. To accomplish this, the 16S rRNA gene fragments were amplified from *S. aureus* strain (ATCC® 29213™) and *S. epidermis* strain (ATCC® 12228™). The primers for 16S rRNA gene amplification were Staphylococci-16S-rRNA-standard-out-F (5'-GTACTCGAGATTTATTGGGCGTAAAGCG-3'), Staphylococci-16S-rRNA-standard-out-R (5'-GCTGGATCCGGGACTTAACCCAACATCTC-3'). The fragment was cloned into pBlueScript II SK+ (Agilent Technologies, Santa Clara, CA) and linearized by XbaI as a DNA template for RNA *in vitro* transcription. Standard RNA template was produced by using Ambion® MEGAscript® kit (Thermo Fisher Scientific, Carlsbad, CA).

## Quantitative Real Time-PCR (qRT-PCR)

### *SYBR green qRT-PCR*

Primers used in SYBR green qRT-PCR assay based on previous studies<sup>25</sup>. 5 µl of the single-stranded cDNA obtained above was analyzed with use of PowerUp™ SYBR Green Master Mix (Thermo Fisher Scientific, Carlsbad, CA). The cycling conditions were 50°C for 10 minutes and 95°C for 5 minutes, followed by 45 cycles of 95°C for ten seconds and 62°C for 30 seconds. For all samples, the threshold cycle number ( $C_T$ ) at which the fluorescence values became logarithmic was determined. The  $\Delta C_T$  value was calculated for each sample as the difference between the sample  $C_T$  and the control  $C_T$ .

### *Standard Curve qRT-PCR.*

Based on the standard RNA prepared from above, total bacterial 16S rRNA copies were quantified via qPCR by using Applied Biosystems™ TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, Carlsbad, CA). The primers and probe were designed based on the *Staphylococcus* 16S rRNA gene consensus region. In this study, we used Staphylococcus-qPCR-F (5'-TCTGTAAGTACGCTGAKGYK-3') as the forward primer and Staphylococcus-qPCR-R (5'-CGAATTAAACCACATGCTCCAC-3') as the reverse primer. The Staphylococcus-16S-rRNA-qPCR-Probe (5'-/56-FAM/CCACGCCGT/ZEN/AAACGATGAGTGCT /3IABkF/-3') was synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa). Briefly, 5 µl of single-stranded cDNA was obtained via Invitrogen™ SuperScript® III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Carlsbad, CA) for qRT-PCR and subjected to qPCR cycling as

follows: 15 minutes at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.

## **Results**

### **Demographics and Description of Study Participants**

A total of 13 patients were identified meeting inclusion and exclusion criteria. Prior to initiating treatment, periprosthetic joint infection was defined by Musculoskeletal Infection Society criteria in 100% of cases. Patients were enrolled after removal of their PMMA spacer. After removal, all antibiotic PMMA spacers did not culture any organisms. The minimum follow up time was 15 months (15-24 months), and no disease recurrence occurred. Preoperative, surgical, and post-operative care was directed by and at the discretion of the patient's medical team. Prior to removal of the antibiotic spacer, all patients had a minimum of 6 weeks of intravenous antibiotic therapy followed by a minimum of 6 weeks of absence of antibiotics. Selection of antibiotics and dosing was determined by the primary clinical team as a unique, optimized function of the microbe antibiotic sensitivity and patient comorbidities and tolerance. Removal of the antibiotic spacer and placement of final components was determined by the surgical team based on ESR, CRP, and, in some cases, synovial cell count.

### **Bacterial 16S rRNA detection demonstrates considerable bacterial contamination of spacers**

To determine if bacterial rRNA could be detected on antibiotic PMMA spacers, we used PCR and RT-PCR with universal 16s primers. Bacterial 16s rRNA gene or bacterial rRNA could be detected in 7 samples of the 13 samples (53.8%; Table 2). We then attempted to semi-quantify

bacteria 16s rRNA levels on PMMA spacers using qRT-PCR. A separate set of primers were used in these experiments, optimized for qRT-PCR (Table 3). The mean threshold cycle number ( $\Delta C_T$ , the difference between the sample  $C_T$  and the control  $C_T$ ) using universal primers was  $27.63 \pm 4.27$  (minimum: 15.3; maximum: 31.6). The  $\Delta C_T$  for sample 1, 3, 10 and 11 were high, indicating a considerable amount of 16S rRNA existed on these spacer samples. Control PMMA spacers were included in all of these experiments. Standard PCR controls were used including treating samples with DNase to remove residual DNA contamination and withholding reverse transcriptase from samples as a negative control, and negligible contamination was observed.

In this study, a qRT-PCR standard-curve method was designed to quantify bacteria 16s rRNA copies from spacer samples (Figure 1). High 16S rRNA copies were observed in 30.8% of samples (4/13) suggesting these samples may contain viable bacteria. Control PMMA spacers were included in all of these experiments.

### **Single Genome Analysis Identified Bacteria on the Spacer Identical to the Initial Cultured Organism**

Single genome analysis was used to identify the species of bacteria present due to the qRT-PCR signal (Table 2). Using qRT-PCR 16s rRNA universal primers (Table 1), a bacterial species could be identified in 53.8% of spacers (7/13). In PJI, 7% to 34% of cases are reported to be culture negative<sup>26-28</sup>. When cases that were culture negative at the initiation of treatment were excluded, SGA identified the same species as initial cultures prior to initiating treatment in 40%

of cases (4/10). Of those detected, many samples yielded mono- and polymicrobial bacterial communities including skin commensals and pathogens 71.4% (5/7).

### **16S rRNA Degradation**

An *in vitro* assay was used to quantify rRNA degradation to test the assumption that rRNA would not be present for more than one month after an initial heat shock treatment. Viable bacteria capable of reproduction on blood agar from the biofilm were quantified. The qRT-PCR standard curve was used to quantify remaining copies of rRNA. At day 28, the bacterial 16S rRNA became undetectable (Figure 2).

### **Discussion**

In this study, we found evidence suggesting viable bacteria remain on antibiotic PMMA spacers after long-term antibiotic treatment in PJI. Bacteria with a phenotypic high tolerance to antibiotics are one of the properties of bacterial persisters, and bacteria persister cells are believed to be a source of chronic or recurrent infections. There are multiple *in vitro* examples demonstrating biofilm cells have a high tolerance to antibiotics as compared to planktonic bacteria<sup>29-31</sup>, but there is a lack of direct clinical evidence supporting this observation. In this clinical study, qRT-PCR was used to determine if viable bacteria remained on PMMA spacers retrieved after antibiotic treatment for TKA PJI. During a two-stage revision for PJI, an antibiotic PMMA spacer is removed after there is no additional evidence of remaining infection for placement of final arthroplasty implants. High levels of bacterial 16S rRNA present on these PMMA spacers suggest viable bacteria remain on these PMMA spacers. The presence of viable

bacteria on PMMA spacers after an extended course of intravenous antibiotics with no evidence of continuing clinical infection provides strong *clinical* evidence of biofilm antibiotic tolerance in PJI.

A series of molecular techniques demonstrated the continued presence of bacterial rRNA on the PMMA spacers. Quantitative PCR has been used for bacterial identification in many conditions, including sepsis and meningitis<sup>32-36</sup>. Using RT-PCR and semi-quantitative RT-PCR, we found a signal was present on all samples. The strong signal present on some samples provides evidence of the presence of bacteria rRNA on the PMMA spacer; however, these methods are binary with the potential for a high false positive rate, especially by using bacterial universal primers.

Absolute quantification by the standard-curve method is frequently used in environmental microbiology<sup>37</sup>. We designed a standard-curve to directly quantify copies of bacteria rRNA. Of these samples 30.8% (4/13) had high 16S rRNA copies suggestive of remaining viable bacteria (Figure 1). These results corroborated our PCR and semi-qRT-PCR results. Negative signal could be the result of three possibilities including a lack of remaining viable bacteria, a primer with insufficient specificity for the low quantities of biofilm, or the signal may have been compromised during preparation from a series of freeze thaw cycles. We modeled our standard curve primer based on the reference genome for *S. aureus*, but with a low biofilm mass, there could be a divergence of results between different bacteria strains based on primer specificity.

Single genome analysis provided a second piece of evidence supporting viable bacteria on retrieved PMMA spacers. In patients that were initially culture positive, we were able to isolate

rRNA that corresponded with the initial cultured bacteria species prior to the initiation of treatment on 44% (4/9) of the PMMA antibiotic spacers. In the remaining 5 cases where SGA did not agree with initial culture, there were 4 samples where SGA was unable to detect a species. This suggests that either viable bacteria were not present, rRNA on the sample had been lost to repeated freeze-thaw cycles, or we did not have a specific enough PCR primer to amplify a low signal for that specific bacterial strain. In the one sample where SGA detected a species that did not agree with initial culture results, the species had a relatively similar antibiotic susceptibility pattern. The disagreement in speciation could be the result of either a contaminant on sample processing or detecting a possible polymicrobial infection where the initial cultured organism was not detected. In clinical studies, multispecies biofilm may contribute to wound chronicity<sup>38</sup>, intracellular infection in otitis media<sup>39</sup> and diabetic chronic wounds<sup>40</sup>. Our data support that the multispecies microbial biofilm may contribute to chronic PJI.

An important limitation of this study is the possible high false positive rate of 16s qRT-PCR. To limit this possibility, we tested the accuracy of the assumption that rRNA would have limited stability making it an appropriate marker for bacteria viability. Antibiotics had been discontinued for a minimum of 6 weeks prior to removal of the PMMA spacer. In our RNA degradation assay, at day 28, the 16S rRNA became undetectable (Figure 2). In other studies, rRNA has been used to detect viable bacteria with stability less than one week<sup>41</sup> and a weak to non-existent signal at 3 weeks<sup>18</sup>. Degradation of RNA plays a central role in RNA metabolism. Messenger RNA (mRNA) decay and stable RNA degradation generally have been considered distinct and separate from RNA maturation<sup>19</sup>. rRNA is more stable than mRNA and believed to have a stability on the

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order of days <sup>42</sup>. rRNA is generally not degraded during exponential growth, but extensive degradation of these molecules occurs under other physiologic conditions <sup>43</sup>. Our *in vitro* assay agrees with this previous literature, however possess the same limitations in correlating an *in vitro* assay with *in vivo* observations. If rRNA degradation occurs on an antibiotic spacer in the treatment of PJI at a similar rate as measured with our *in vitro* assay, then the identified bacterial 16s rRNA could have come from only viable bacteria.

Biofilms play an important role in medical device infections based on their inherent resistance to antibiotic chemotherapy <sup>44</sup>. The continued presence of bacterial rRNA on PMMA spacers suggests that viable bacteria remain after explant of implants and extended systemic antibiotic therapy had been discontinued in PJI. This study extends *in vitro* observations that biofilms are highly tolerant to antibiotics <sup>45</sup>. This suggests that subclinical biofilm cells remains after treatment and provides evidence for the importance of the host immune system at either removing or controlling any remaining biofilm. In other words, our data suggests that a two-stage revision for PJI does not completely remove the infection, but is a critical step in helping the host control the infection. In a two-stage revision for PJI, approximately 15% of patients are unable to sustain a reimplantation of components, and a complete two-stage revision has a treatment failure rate between 10-20% (PMID: 26378265). In these patients, our findings suggest at least a piece of the mechanism behind PJI treatment failure is the inability of antibiotics to completely remove the biofilm and provides evidence for considering the utility of long-term antibiotic suppression to control the infection in select patients at high risk for treatment failure.

### **Acknowledgements:**

The study was supported in part by funding from the National Institutes of Health (NIH) through the grants K08 AR071494 (KLU), KL2 TR000146 (KLU), KL2 TR001856 (KLU), and 5 UL1 TR0000005 (KLU, RMS) from the National Center for Advancing Translational Sciences (NCATS). Additional funding support was provided in part by the Woodward Family Endowment in Biomedical Engineering (CMD, TKW, KLU) and the Pellegrini Endowment to the Department of Orthopaedics and Rehabilitation at the Pennsylvania State University (KLU).

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Table 1. Universal bacterial 16S rRNA primers.

| Primer          | Sequence                | Reference                            |
|-----------------|-------------------------|--------------------------------------|
| 16S-rRNA-27-F   | AGAGTTTGATYMTGGCTCAG    | Turner et al. 1999 <sup>46</sup>     |
| 16S-rRNA-337-F  | ASACTCCTACGGGRSGCWGCART | Greisen et al. 1994 <sup>47</sup>    |
| 16S-rRNA-533-F  | GTGCCMGCMSCCGCGGTAAT    | Weisburg et al. 1991 <sup>48</sup>   |
| 16S-rRNA-785-F  | GGATTAGATASCCTGGTAGTC   | Klindworth et al. 2013 <sup>49</sup> |
| 16S-rRNA-336-R  | GTATTACCGCGGCKGCTG      | Greisen et al. 1994 <sup>47</sup>    |
| 16S-rRNA-518-R  | ATTACCGCGGSKGCKGGCAC    | Ferris et al. 1996 <sup>50</sup>     |
| 16S-rRNA-536-R  | GTATTACCGCGGCKGCTG      | Dahllof et al. 2000 <sup>51</sup>    |
| 16S-rRNA-805-R  | GACTACCAGGSTATCTAATCC   | Tanner et al. 1999 <sup>52</sup>     |
| 16S-rRNA-926-R  | CCGTCAATTCMTTTGAGTTT    | Klindworth et al. 2013 <sup>49</sup> |
| 16S-rRNA-1406-R | ACGGGCGGTGTGTRCAA       | Youssef et al. 2009 <sup>53</sup>    |
| 16S-rRNA-1492-R | CGGYTACCTTGTTACGACTT    | Turner et al. 1999 <sup>46</sup>     |

Table 2 The bacteria strains detected by SGA in the spacer samples

| Sample ID | Initial Culture species           | Strain-1                     | Strain-2                        | Strain-3             | Match with initial culture species(Y/N) |
|-----------|-----------------------------------|------------------------------|---------------------------------|----------------------|---|
| 1         | <i>Staphylococcus aureus</i>      | <i>Staphylococcus aureus</i> | <i>Acidovorax</i>               | <i>Acinetobacter</i> | Y                                       |
| 2         | <i>Staphylococcus aureus</i>      | <i>Staphylococcus aureus</i> | <i>Acinetobacter</i>            | <i>Bacillus</i>      | Y                                       |
| 3         | Culture Negative                  | Uncultured bacterium         |                                 |                      | Y                                       |
| 4         | Culture Negative                  | Undetected                   |                                 |                      | Y                                       |
| 5         | <i>Staphylococcus epidermidis</i> | <i>Streptococcus</i>         | <i>Pseudomonas</i>              |                      | N                                       |
| 6         | <i>Staphylococcus epidermidis</i> | Undetected                   |                                 |                      | N                                       |
| 7         | <i>Cornyebacterium</i>            | Undetected                   |                                 |                      | N                                       |
| 8         | Culture Negative                  | Undetected                   |                                 |                      | Y                                       |
| 9         | Culture Negative                  | <i>Streptococcus</i>         | <i>Saccharomyces Cerevisiae</i> |                      | N                                       |
| 10        | <i>Staphylococcus aureus</i>      | <i>Staphylococcus aureus</i> | <i>Streptococcus</i>            |                      | Y                                       |
| 11        | <i>Staphylococcus aureus</i>      | <i>Staphylococcus aureus</i> |                                 |                      | Y                                       |
| 12        | <i>Staphylococcus</i>             | Undetected                   |                                 |                      | N                                       |
| 13        | <i>Staphylococcus epidermidis</i> | Undetected                   |                                 |                      | N                                       |

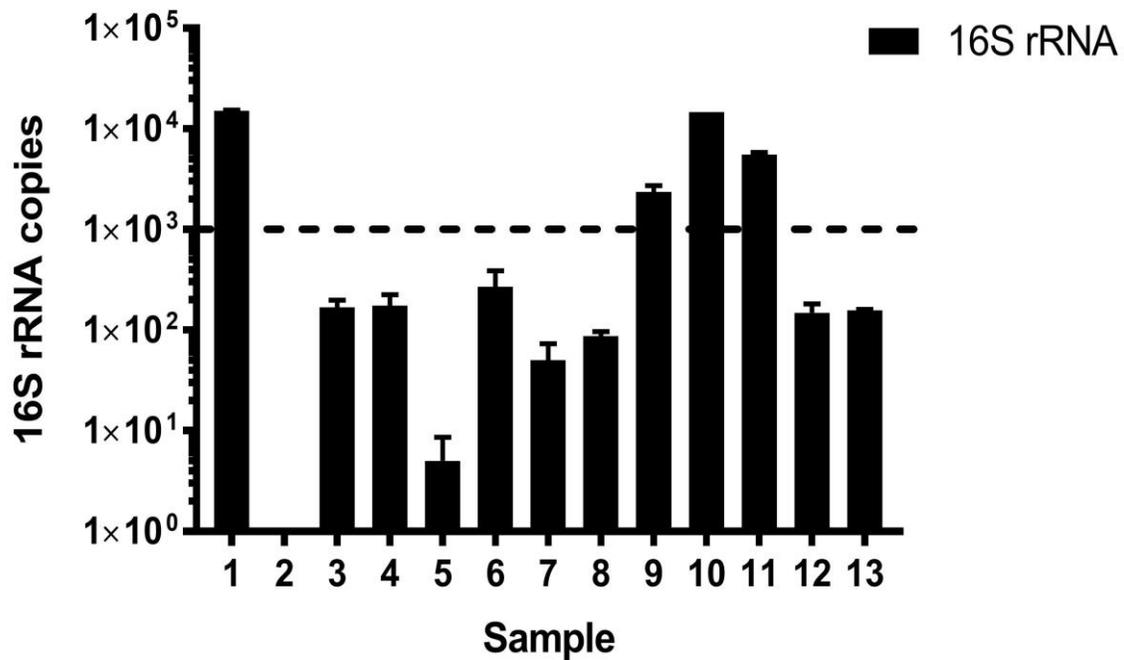
Table 3. qRT-PCR results of different sets of primers ( $\Delta C_T$ ) and absolute numbers

| Sample ID | qPCR-set1 ( $\Delta C_T$ ) | qPCR-set2 ( $\Delta C_T$ ) | qPCR-set3 ( $\Delta C_T$ ) | qPCR-Universal ( $\Delta C_T$ ) | 16S rRNA copies | STD curve control |
|-----------|----------------------------|----------------------------|----------------------------|---------------------------------|-----------------|-------------------|
| 1         | 28.6                       | Undetermined               | Undetermined               | 25.8                            | 15017           | Undetermined      |
| 2         | 31.7                       | Undetermined               | Undetermined               | 29.3                            | 0               | Undetermined      |
| 3         | 27.6                       | 28.4                       | 25.1                       | 15.3                            | 168             | Undetermined      |
| 4         | 28.9                       | 26.1                       | 29.9                       | 31.6                            | 174             | Undetermined      |
| 5         | 36.9                       | Undetermined               | Undetermined               | 29.8                            | 5               | Undetermined      |
| 6         | 31.2                       | Undetermined               | Undetermined               | 29.4                            | 270             | Undetermined      |
| 7         | 33.4                       | Undetermined               | Undetermined               | 29.7                            | 50              | Undetermined      |
| 8         | 36.6                       | Undetermined               | Undetermined               | 29.6                            | 86              | Undetermined      |
| 9         | 28.8                       | Undetermined               | Undetermined               | 30.4                            | 2366            | Undetermined      |
| 10        | 19.6                       | Undetermined               | Undetermined               | 24.4                            | 14567           | Undetermined      |
| 11        | 31.9                       | Undetermined               | Undetermined               | 25.3                            | 5558            | Undetermined      |
| 12        | 32.5                       | Undetermined               | Undetermined               | 29.7                            | 148             | Undetermined      |
| 13        | 29.6                       | Undetermined               | Undetermined               | 28.9                            | 157             | Undetermined      |

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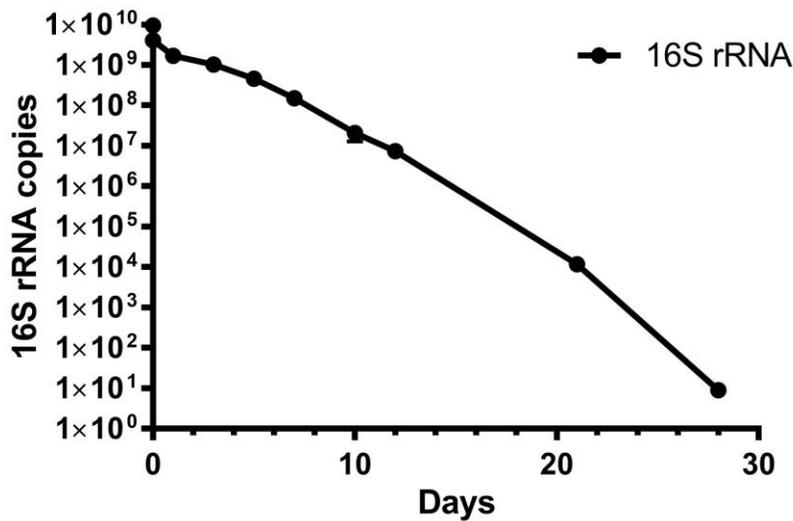
## Figure Legends

Figure 1. 16S rRNA copies in clinical spacer samples. Based on the standard RNA we prepared, a qRT-PCR standard curve was determined for bacterial 16S rRNA quantification. From the qRT-PCR standard curve, the quantity of 16S rRNA copies were calculated. 15,014, 2,366, 14,567 and 5,558 16S rRNA copies were detected from samples 1, 9, 10 and 11, respectively.



**Figure 1 16S rRNA copies in clinical spacer samples**

Figure 2. Bacterial 16s rRNA degradation. Titanium rods were incubated in TSB growth medium inoculated with  $1 \times 10^6$  CFU/ml *Staphylococcus aureus* (SH1000) for 48 hours (biofilm). The rods were sonicated for 20 minutes and centrifuged at 8000 rpm for 5 minutes to collect the bacteria. The bacteria were lysed by lysozyme solution with RNase A added, followed by proteinase K/SDS treatment. Samples were stored at 37°C for up to 28 days. At each time point, total RNA extraction was performed for subsequent qRT-PCR analysis. At day 28, the bacterial 16S rRNA became undetectable.



**Figure 2 Bacterial 16S rRNA degradation-Lysozyme treatment**