

Staphylococcus strains show resistance to antibiotics in both in-vitro and in-vivo settings

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ABSTRACT

Hospitals often use *in vitro* methods to assess the susceptibility of bacterial strains and use the information collected to guide physicians in the selection of suitable antibiotics to treat infected patients. Much research has already shown that *in vitro* methods can lead to false conclusions of bacterial susceptibility since bacteria grown planktonically are hundreds or thousands of times more sensitive to antibiotic treatment compared to bacteria growing in a biofilm. For this study, we used a rat subcutaneous implant model to test this concept by directly challenging “sensitive” and “resistant” *Staphylococcus* strains first *in vitro* and then *in vivo*. As anticipated, both “sensitive” and “resistant” bacteria were immune to extremely high levels of antibiotic therapy when they have contaminated a device implant. These results confirm the necessity of using *in vivo* animal models such as this rat subcutaneous implant model for testing the efficacy of new antibiotics particularly when those antibiotics are to be developed to treat device-related infections and/or antibiotic resistant organisms.

METHODS

Preparation of Inoculum:

Two days prior to the experiment, streak a small loopful of test organisms from frozen culture collections and culture on non-selective agar plates (e.g. TSA). Incubate overnight at 37°C ± 1°C and examine the cultures for purity prior to use.

Test organisms:

- Methicillin-resistant *Staphylococcus aureus* (ATCC 33592)
- Staphylococcus aureus* (CBE-71)
- Staphylococcus aureus* (6538)
- *Staphylococcus aureus* (CNS)

Incubate at 37°C ± 1°C for approximately eighteen hours.

Preparation of each inoculum:

The day prior to the experiment a small loopful of each microorganism from the above-mentioned strains were placed into full strength Mueller Hinton Broth and vortexed for in-vitro study using all above-mentioned strains, and into Tryptic Soy Broth and vortexed for additional two follow-up in-vivo studies. Tubes were then incubated in a shaker at 37°C ± 1°C for approximately eighteen hours.

Kirby- Bauer macrodilution method:

The preliminary in-vitro study used the Minimum Inhibitory Concentration (MIC) tube dilution method. Beginning with the stock solution of 500 mg/mL Mupirocin in MHB, 500 mg/mL Methicillin in MHB, or 500 µg/mL Gentamycin in MHB in the first tube, serial doubling dilutions of 1:1 were performed through 12 tubes with additional MHB solution. Each set was completed in triplicate and a negative control with no active agent was also prepared. To each tube, sufficient bacterial inoculum was added to yield a starting population of approximately 10⁵ CFU/mL. The tubes were incubated for approximately 24 h at 37°C ± 1°C and scored the tubes for their MIC.

Scoring MIC tubes:

The highest dilution (lowest concentration of active) not demonstrating visible outgrowth (turbidity) in three replicates, as assessed by the unaided eye will be considered the MIC.

A table was used to indicate whether each of the triplicate tubes is clear or turbid.

In-vivo assessment of antimicrobial efficacy of Methicillin and Mupirocin

The first follow-up in-vivo study used young male Sprague-Dawley rats that were anesthetized, shaved, and wiped with 70% isopropyl alcohol. A 2 cm subcutaneous pocket was created and a Surgipro mesh was inserted into the space with 0.1 mL of 10⁷ *Staphylococcus aureus* immediately following. The wound was then sealed with tissue glue and pain medication given immediately and again on day 1. On days 2 and 3, treatment was given IM as follows: Test group 1: MRSA treated with 5mg/Kg (2mg/rat) Methicillin; Test group 2: MRSA treated with 5mg/Kg (2mg/rat) Mupirocin; Test group 3: 6538 treated with 5mg/Kg (2mg/rat) Methicillin; Test group 4: 6538 treated 5mg/Kg (2mg/rat) Mupirocin; Control/ Infected Group 5: 6538 with no treatment; Control/ Infected group 6: MRSA with no treatment. On day 4, rats were euthanized and wound and mesh assessed for infection, tissue in-growth, and disintegration. A second follow-up in-vivo study was completed in the same manner as above with the following changes: Rats were treated twice daily on days 2,3, 4, and 5, and sacrificed on day 6.

In-vivo sampling

Entire mesh was removed from the pocket and placed in a sterile petri dish with 1.0 mL neutralizing buffer, scraped with a sterile blade, sonicated for ten minutes, serial diluted, and drop plated on TSA and MSA plates. Additionally, surrounding tissue was collected and placed in pre-weighted microcentrifuge tubes containing neutralizing buffer, weighed to determine tissue weight, homogenized and sonicated for ten minutes, serial diluted and drop plated on TSA and MSA plates.

Calculations:

Use dilutions giving 30 to 300 colonies per plate. Multiply colonies counted by inverse of dilution and inverse of fractional volume plated.
CFU/mL = (dilution factor)⁻¹ X (correction factor)⁻¹ X average number of colonies
EXAMPLE: If the 10⁻³ dilution gives an average plate count of 42 colonies per plate, and 100µL volumes were plated, then:
CFU/mL = (10⁻³)⁻¹ X (0.1)⁻¹ X 42
= 1000 X 10 X 42
= 4.2 X 10⁵

RESULTS

In the in-vitro study, the MRSA strain was consistently more resistant to antibiotic challenge for all three of the antibiotics assessed with the increased resistance ranging from 10x more than 100x (Figure 1, below).

By contrast, both of the in-vivo studies showed that although 6538 and MRSA grew to similar levels on the implanted meshes and in the surrounding tissues, both were equally resistant to both antibiotics in the in vivo environment (Figures 2-5, below and right).

Figure 1: MIC Summary Data

Table 1. Mupirocin MIC Summary Data	
ATCC 6538	Less than 0.24ug/mL
CBE 71	0.48 ug/mL
CNS	0.48ug/mL
MRSA	1.95 ug/mL

Table 2. Gentamicin MIC Summary Data	
ATCC 6538	3.9 ug/ml
CBE 71	3.9 ug/ml
CNS	1.95ug/mL
MRSA	Great than 500 ug/mL

Table 3. Methicillin MIC Summary Data	
ATCC 6538	3.9 ug/mL
CBE 71	125 ug/mL
CNS	15.6ug/mL
MRSA	Great than 500 ug/mL

Figure 2. Once-daily antibiotic treatment for 2 days:
Log CFU's per mesh

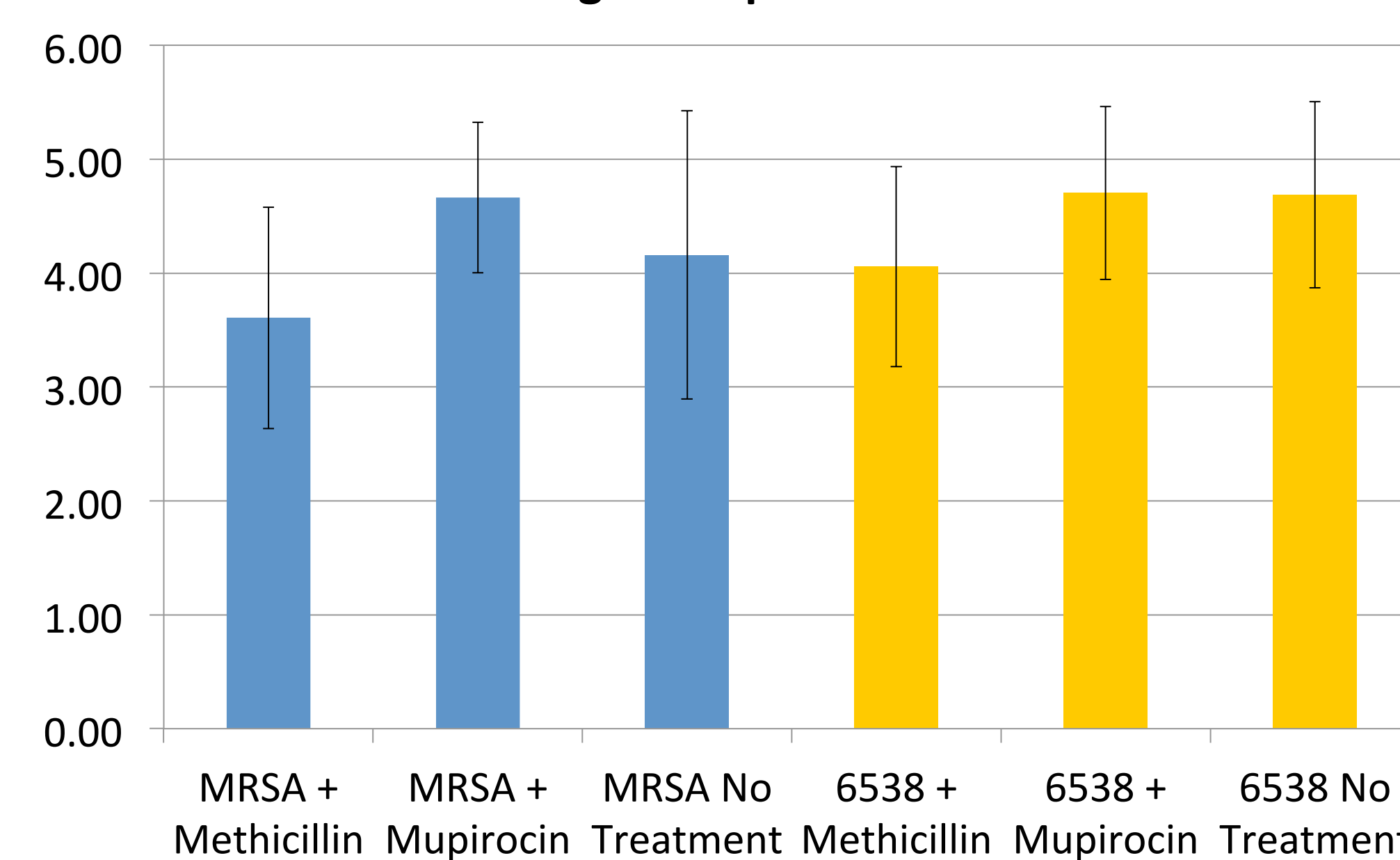


Figure 2:

All the bacteria recovered from the implanted meshes showed similar levels of growth. There were no statistically significant differences between any of the groups of bacteria strain or antibiotic used as determined by an ANOVA.

Figure 3. Once-daily antibiotic treatment for 2 days:
Log CFU's per gram of Tissue

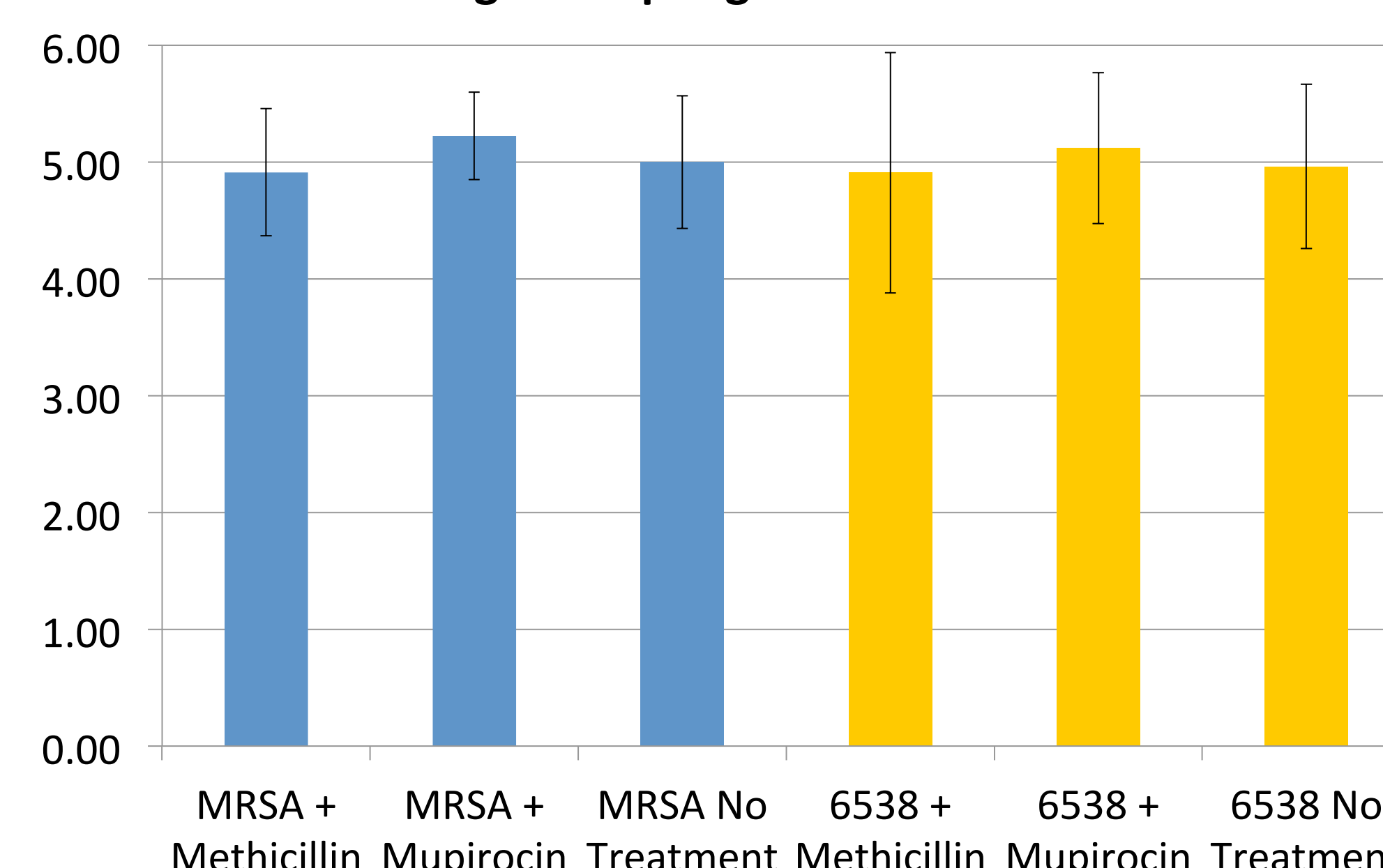


Figure 3:

All the bacteria recovered from the tissues surrounding the implanted meshes showed similar levels of growth. There were no statistically significant differences between any of the groups of bacteria strain or antibiotic used as determined by an ANOVA.

Figure 4. Twice-daily antibiotic treatment for 4 days:
Log CFU's per Mesh

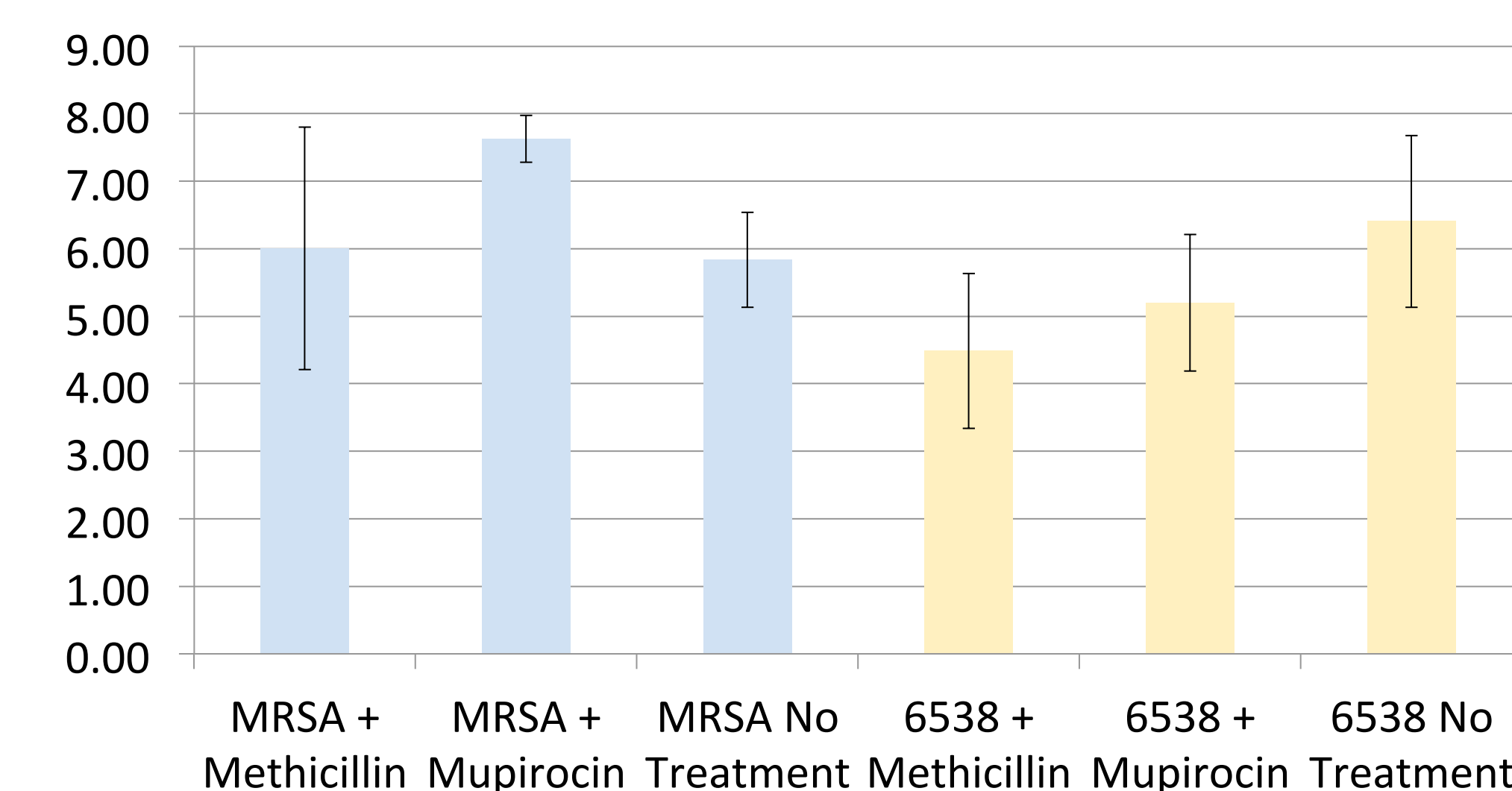


Figure 4:

All the bacteria recovered from the implanted meshes showed similar levels of growth. There were no statistically significant differences between any of the groups of bacteria strain or antibiotic used as determined by an ANOVA.

Figure 5: Twice-daily antibiotic treatment for 4 days:
Log CFU's per gram of Tissue

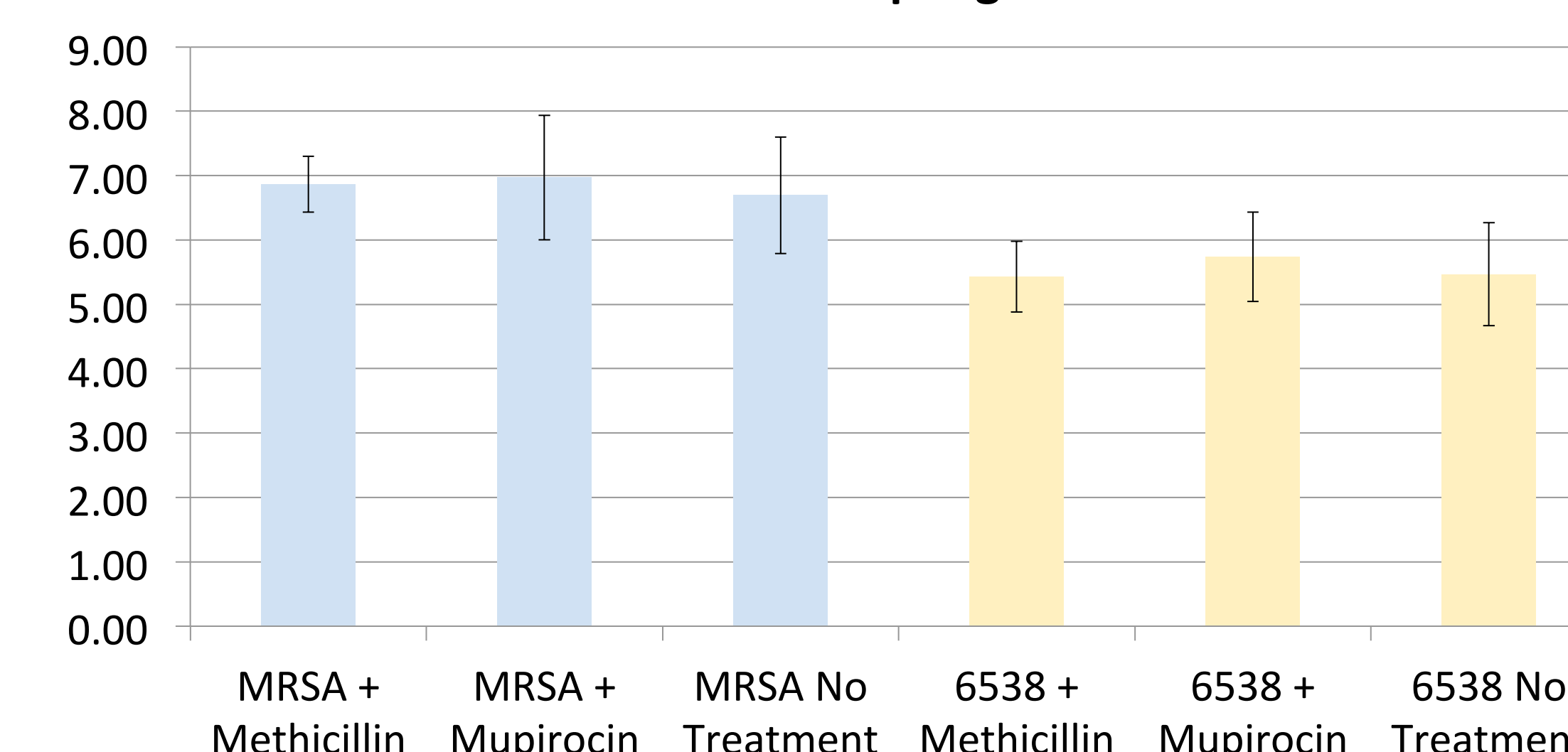


Figure 5:

All the bacteria recovered from the tissues surrounding the implanted meshes showed similar levels of growth. There were no statistically significant differences between any of the groups of bacteria strain or antibiotic used as determined by an ANOVA.

CONCLUSIONS

These results highlight the extreme resistance of biofilm bacteria and demonstrate that terminology such as “resistant” can be misleading when considering actual susceptibility to antibiotics in a clinical (in vivo) setting.

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