# Comparison of Pre-Operative Site Sterilization Techniques in Equine Medicine

A Major Qualifying Project Report
Worcester Polytechnic Institute
Department of Biology and Biotechnology



Advisors:
Mike Buckholt, PhD
Michael Myhre, DVM
Jill Rulfs, PhD

Author: Holly Galvin

# Abstract

In order to limit post operative infections, surgical preparation is an important step to limit exposure to microorganisms. The goal of this project was to compare the two different pre-procedural sterilization protocols to determine if one was more effective at lowering colony forming units cultured from the sterilization site on equine patients. Both sterilization methods were effective in reducing colony forming units and there was no significant difference observed between the two sterilization protocols. Both sterilization methods had mostly *Bacillus* left over after sterilization.

# Acknowledgments

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# 1: Introduction and Background

## 1.1: Post Surgical Infections

Post operative infections affect patient outcomes after surgery for both human and veterinary patients. Infections can range from those easily treated by antibiotics to fatal complications. In order to limit post operative infections, aseptic technique is used to limit exposure to microorganisms. Aseptic technique includes three main tools for preventing contamination. Physical barriers between patient and doctor are used such as sterile gloves, gowns, masks, hairnets, and drapes. Once the physical barriers have been put into place, it is important for the surgeon to only touch other sterile items, such as surgical equipment. Environmental controls are also used, such as limiting the people in the surgical room or properly filtering the air in the room. The final important step is thoroughly prepping the surgical area with antiseptic scrub to kill any bacteria already in the area (Wikstrom, 2019).

Even with surgeons following careful aseptic guidelines, post surgical infections still occur as it is impossible to reach 100% sterility on the skin. In equine medicine specifically, reports of surgical site infections (SSIs) range from 0.7-50% of all patients, depending on the procedure performed (Ahern, 2012). In a study exploring SSI cases among 846 canine patients discharged after veterinary medical procedures, clients were contacted with a survey 30 days post operation. Researchers found that 35% of the 26 postoperative infections reported in the survey were not recorded in the medical record (Turk, 2015). This indicates that the scope of post surgical infections may be larger than what is often reported and seen at veterinary clinics. From a study conducted at the Koret School of Veterinary Medicine on the most common postoperative infections in equine patients, the most common bacteria found were methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (Kelmer, 2020).

## 1.2: Factors Increasing Likelihood of Post Surgical Infections

Different factors can increase the likelihood of an equine patient developing a post surgical infection. Abdominal procedures, such as colic surgery, have the highest surgical site infection rates, due to the length and invasivity of the procedure. Other factors increasing the likelihood of developing infection in equine patients include pregnancy, weight problems, and repeat surgeries (Kelmer, 2020). Longer surgical duration and time in the hospital has also been shown to increase SSIs in equine patients (Ahern, 2012).

Non-compliance with proper recommended aseptic procedures is also an issue contributing to postoperative infections. A survey of medical doctors found that 63% did not comply with recommended procedures for pre-surgical scrubbing in (Davis, 2008). In an observational study looking at ten different small animal veterinary clinics, researchers found that though the recommended pre-surgical scrubbing time from the AVMA is 2-5 minutes, the median time observed was only 67 seconds. Durations as low as 10 seconds were also reported (Anderson, 2013).

## 1.3: Reagents & Mechanism of Action

Surgical site preparation with proper chemicals for the full duration of the recommended time is very important in preventing post-surgical infections. 0.75% povidone iodine and 70% isopropyl alcohol are two common bactericides used in preparation and this study. Povidone iodine is a broad spectrum antibacterial. It is water soluble, and consists of iodine and polyvinylpyrrolidone, a solubilizing polymer carrier. The polyvinylpyrrolidone delivers the iodine to the surface of the cell, allowing iodine to penetrate the cell and oxidize nucleotides, fatty acids, and protein. This allows it to kill many bacterial species, as well as fungi, protozoa, and viruses (Lacapelle, 2019). In a study comparing 4% chlorhexidine gluconate-alcohol, 1%

p-chloro-m-xylenol, 3% hexachlorophene, and 1% povidone iodine, povidone iodine was found to be the most effective in killing MRSA, the most common cause of post-surgical infections (Haley, 1985). 70% isopropyl alcohol is also a broad spectrum antibacterial. It kills bacteria by denaturing the proteins that give structure to the bacteria, causing the cells to dehydrate and die (Mangram, 1999). Efficacy of 70% isopropyl alcohol and 1% povidone iodine in killing different pathogens can be seen below in table 1. Efficacy of antiseptic is also affected by other factors including concentrations, temperature, pH, humidity, and water hardness (Mangram, 1999).

Table 1: Comparison of 70% isopropyl alcohol and 1% povidone iodine as Antiseptics. Information from: (Mangram, 1999)

| **Agent** | **Mechanism of Action** | **Gram-Positive** | **Gram-Negative** | **Fungi** | **Virus** | **Rapidity of Action** | **Residual Activity** |
|---|---|---|---|---|---|---|---|
| 70% isopropyl alcohol | Denature Proteins | Yes | Yes | Yes | Yes | Rapid | None |
| 1% povidone iodine | Oxidation/ Substitution by free iodine | Yes | Yes | Yes | Yes | Intermediate | Minimal |

There has been minimal difference found between the sterilization efficacy of the 70% isopropyl alcohol and 10% povidone iodine. In a study comparing the disinfectant effect of using both 70% isopropyl alcohol and 10% iodine povidone in different orders of application on human forearms, there was no significant difference found in reduction of bacterial load (Kim, 2013). Using both reagents in combination was more effective than using only one reagent. 76.6% of bacteria were killed with just 70% isopropyl alcohol, 80.5% of bacteria were killed

with just 10% iodine povidone, while 95% of bacteria were killed when both reagents were used in combination (Kim, 2013).

### 1.4: Bacteria Resistant to Antiseptic Agents

There are many qualities of bacterial species which predict resistance to regular sterilization procedures. Anatomical barriers block antiseptics such as 70% isopropyl alcohol or 0.75% povidone iodine from entering the bacteria. Mycobacteria also have a waxy wall that is hard to penetrate as well as the outer membrane of gram-negative bacteria (Russel, 1999). Bacteria with spores such as genus *Bacillus* or *Clostridium* are the most resistant due to the presence of endospores which surround and protect the cell. There are many methods to kill endospores, but they cannot be used in surgical preparation. Some of the most commonly used methods can be seen in Table 2 below.

Table 2: Methods for killing endospores.

| **Method** | **Agent** | References |
|---|---|---|
| High Heat + Pressure | Autoclaving | Huesca-Espitia et al. (2016) |
| Radiation | UV-C (222 nm, 254 nm, pulsed light), | Narita et al. (2020) |
| Chemicals | HCl, NaOH, $ClO^-$, and $H_2O_2$ | Setlow et al. (2002) |

### 1.5: CFUS and Growth Media

In the study reported here, the relationships of colony forming units cultured from pre-surgical skin swabs and post-operative infections were studied to determine if there is a

relationship between the two. In a prospective observational study of 609 human patients, researchers found that higher amounts of colony forming units cultured from pre-surgical swabs taken from the surgical site independently increased the risk of development of post surgical infections in lower limb vascular surgery (Turtainian, 2014). In another study of 226 operation incisions, researchers found a very high correlation between bacteria density during the procedure and post surgical wound sepsis (Raahave, 1990).

In order to compare surgical sterilization methods, plating and growth media were used. Muller Hinton Agar is a common agar used in veterinary clinics for antibiotic susceptibility tests due to its generality and ability to grow a large range of pathogens (NCCLS, 1979). It is also very effective in growing *MRSA* and *Pseudomonas aeruginosa,* common bacteria isolated from post surgical infections in equine patients (NCCLS, 1979). Muller Hinton Agar was also used in the culture and sensitivity testing in the study performed at the Koret School of Veterinary Medicine focusing on factors increasing risk of post-surgical infection (Kelmer, 2020).

### 1.6: Hypothesis

Veterinary clinics are able to choose which chemicals they would like to use to sterilize surgical sites before procedures. At the Myhre Equine Clinic in Rochester, NH, they use an iodine scrub for five minutes followed by 70% isopropyl alcohol wipes until the white wipes no longer have visible iodine when wiped on the area. Another common technique used in other clinics is to sterilize using the same regents, but instead in rounds alternating between iodine and alcohol for five minutes.

The goal of this project was to compare the two different pre-procedural sterilization protocols to determine if one was more effective at lowering colony forming units cultured from the sterilization site. The primary literature indicates minimal differences between the two

antiseptics as discussed in Section 1.3, so the duration and order of usage should not affect overall bacterial growth. The hypothesis of this project was that there would be no significant difference in the effectiveness of the two sterilization procedures in reducing colony forming units. The hypothesis would be rejected if:

1. There was a statistically significant difference in the mean number of bacterial colonies cultured from collection swabs taken from equine patients after five minutes of each sterilization protocol.
2. The bacteria colonies sequenced from each sterilization method were significantly different in species composition.
3. There was a significant difference in the mean number of colonies cultured from collection swabs taken from pig skin induced with *Staphylococcus epidermidis* (safe relative of MRSA) and *Pseudomonas aeruginosa putida* (safe relative of *Pseudomonas aeruginosa*) after five minutes of each sterilization protocol.

There was no significant difference observed between the two sterilization protocols for any of the experiments and the hypothesis was accepted. There was no significant difference in the mean number of bacterial colonies cultured from collection swabs taken from equine patients after five minutes of each sterilization protocol for any collection position on the equine patient. There was not a significant difference in the bacterial species composition between the two methods as the bacteria left over from both sterilization methods were mostly *Bacillus.* Finally, there was no significant difference in the mean number of colonies cultured from collection swabs taken from pig skin induced with *Staphylococcus epidermidis* (safe relative of MRSA) and *Pseudomonas aeruginosa putida* (safe relative of *Pseudomonas aeruginosa*) after five minutes of each sterilization protocol.

# 2: Methods and Materials

2.1: Comparison of Two Sterilization Techniques on Equine Patients

**Animals -** Healthy lesson horses owned by High Knoll Farm as well as long term Myhre Equine Clinic residents were used in this study. There were 7 different horses (*Equus caballus*) from a range of breeds and ages.

Table 3: Ages and breeds of live horses used in the study.

| **Horse** | **Breed** | **Age** |
|---|---|---|
| #1 | Thoroughbred | 7 |
| #2 | American Warmblood | 25 |
| #3 | Oldenburg | 8 |
| #4 | American Paint | 14 |
| #5 | Appendix Quarterhorse | 20 |
| #6 | Quarterhorse | 15 |
| #7 | Oldenburg | 17 |

**Sample Collection -** The horses were shaved in the same position on opposite sides of their body across the medial plane. The shaved spots were 1 inch x 1 inch in size, and shaved with a 40 clipper blade. An example of a collection site can be seen in Figure 1 below.

Figure 1: Example of a shaved sacroiliac collection site pre-sterilization.

Collection spots of interest were chosen to model different surgeries and procedures commonly performed on horses. This included the area around the lateral palmar digital nerve on the back of the pastern (neurectomy), the carpus (arthroscopy), the hawk (hawk injection), the midline (colic surgery), and the sacroiliac (SI injection). The spots can be seen in Figure 2 below.

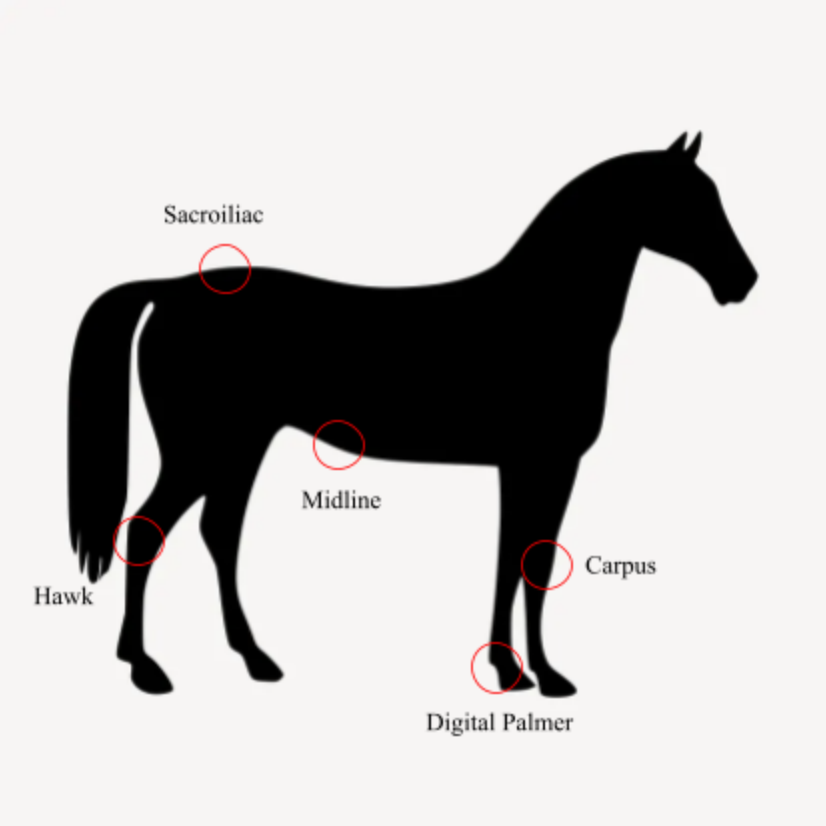


Figure 2: Collection spots on each equine patient. Each spot was repeated twice in the same area across the medial plane of the horse.

A 6-inch long dry cotton sterile swab was used to collect control samples from the shaved areas to begin. The sterile swab was swiped across the shaved area 10 times back and forth starting from the top left corner to the bottom left corner of the area with the head of the swab directly on the skin. The swabbing pattern was kept the same throughout all sample collections, and can be seen below in Figure 3.

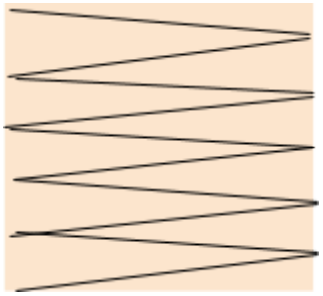

Figure 3: Swabbing pattern used to collect bacterial samples throughout the whole experiment.

Two separate sterilization protocols were used in this study. Both protocols used Covetrus brand 0.75% povidone iodine medical scrub on non-sterile 4 ply 4x4 non-woven sponges, as well as Covetrus brand 70% isopropyl alcohol on non-sterile 4 ply 4x4 non-woven sponges. The first sterilization method was to scrub with the 0.75% povidone iodine wipes for five minutes, and finish with 70% isopropyl alcohol wipes until the alcohol wipes no longer had orange iodine come off of the collection area, as seen below in figure 4.

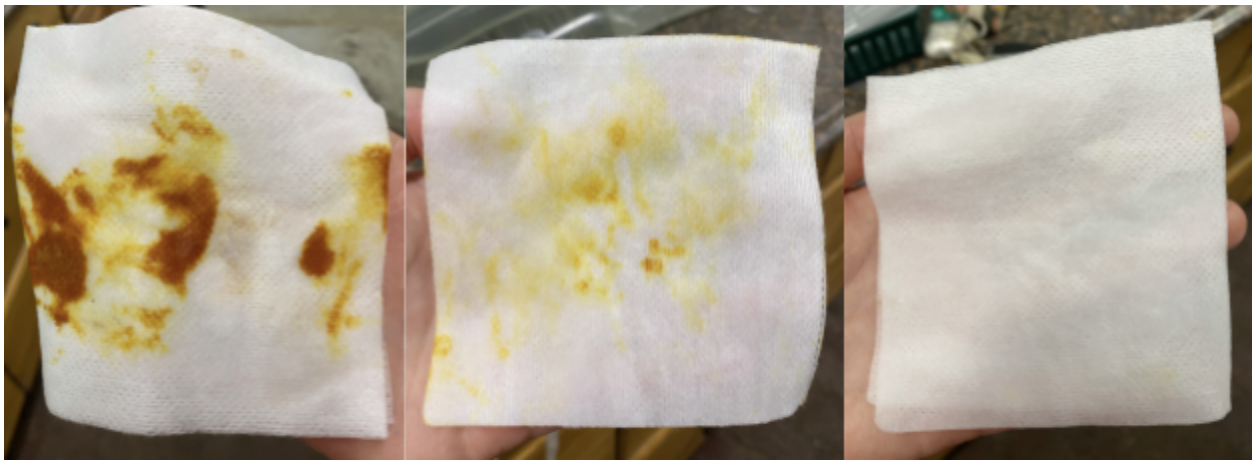

Figure 4: Progression of the coloration of 70% isopropyl alcohol wipes during sterilization method #1

The second sterilization method was to alternate every 15 seconds between 0.75% iodine povidone solution wipes and 70% isopropyl alcohol for five minutes. The side of the horse that each method was performed on was decided by a coin flip, and one method was used on one spot while the other was used on the same location across the midline. Each method was timed using a timer and paused at 30 seconds, 1 minute, and the full five minutes to be swabbed with a sterile swab using the same standard pattern detailed above in Figure 3. The swabs were kept in Sterile Vacuette 9 ml no additive tubes for 20-120 minutes depending on collection time.

Muller Hinton agar plates were divided into four quadrants, with one quadrant for the control taken prior to any sterilization, 30 seconds of sterilization, 1 minute of sterilization, and five minutes of sterilization. Each sample was streaked on the plates in the designated area, which can be seen below in Figure 5. The plates were placed in the incubator at 37 degrees celsius for 48 hours.

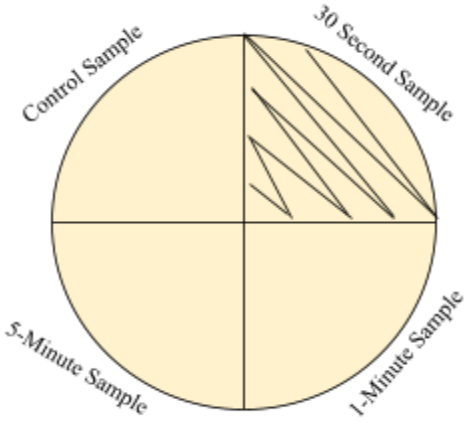


Figure 5: Quadrant plates set up with designated area for the four samples taken from each round of testing, and an example of the plate streaking pattern.

**Quantification of Colony Forming Units -** After incubation, the colonies on the plates were counted for each sample and recorded in an Excel spreadsheet. The samples were quadrant plated on petri dishes ten centimeters in diameter, so colony forming units were counted in colonies per 20 square centimeters. Sections with too many colonies to count were observed on the control samples, and this was indicated in the data to provide an indication of bacteria growth prior to sterilization.

**Statistical Analysis -** A matched pair sample t-test was performed to see if there was a difference between the mean amount of colonies left over after five minutes of sterilization from the two separate methods. There were 35 samples from each sterilization method. The t-value was found using the following equation. $t = \frac{(\sum D)/N}{\sqrt{\frac{\sum D^2 - (\frac{(\sum D)^2}{N})}{(N-1)(N)}}}$ where D = difference in the amount of colonies counted between the two sterilization protocols on collection sites across the midline of the horse, and N = number of samples collected. In addition, the mean and standard error of the mean was calculated from all sample collection areas using the following equation. $MOE = 1.960(s/\sqrt{N})$ where s is the standard deviation from the mean, and N is the number of trials collected.

2.2: PCR on Colonies Left Over after 5-minutes of Sterilization

**Picking Colonies:** The plates used in Section 2.1 were collected from the incubator. A sterile toothpick was used to pick each colony present after the full five minute sterilization protocols

and transfer them to a master Muller Hinton Agar plate. All the colonies that were isolated on the plates without contamination from other colonies were picked. Two master plates were created, one for each sterilization method.

**Lysing the Bacteria:** 400 microcentrifuge tubes were obtained and labeled to indicate which master plate they were picked from. 200 µl of pure water was pipetted into each tube. A small pipette tip of one colony was picked and placed in one tube. This was repeated for every colony. The PCR tubes were then placed in the thermocycler. The thermocycler was set to run 3 rounds alternating between 10 minutes at 94 degrees celsius and 1 minute at 4 degrees celsius to lyse the bacteria.

**PCR Reaction:** 2 µl of each sample of lysed bacteria was pipetted into separate pcr bead tubes filled with 10 µl of 2X OneTaq master mix. 6 µl of sterile $dH_2O$ was then pipetted into each tube. 2 µl of a mixture of 0.2 µM 27F forward primer and 0.2 µM 1492R reverse primer was pipetted into each tube. Composition of both primer sequences can be seen in Table 4 below.

Table 4: Composition of Both Forward and Reverse Primers Used

| 27F Primer | 1492R Primer |
|---|---|
| 5'-AGA GTT TGA TYM TGG CTC AG-3' | 5'-TAC GGG TAC CTT GTT ACG ACT T-3' |
| 5'-AGA ATT TGA TCT TGG TTC AG-3' | |
| 5'-AGA GTT TGA TCC TGG CTT AG-3' | |
| 5'-AGG GTT CGA TTC TGG CTC AG-3' | |

The tube was gently vortexed on the lowest setting. The tubes were then put into the thermocycler. The PCR reaction begins by heating the sample to denature the DNA into two separate strands of single-stranded DNA. The forward primer binds to the template DNA and the reverse primer binds to the complementary strand of DNA to amplify the DNA between positions 27 and 1492 of bacterial 16S rRNA genes. This process is repeated many times to create a large quantity of DNA to be sent for sequencing. The cycling parameters can be seen below in Table 5.

Table 5: Thermocycler Setting

| | |
|---|---|
| 95 degrees Celsius | 60 seconds |
| 95 degrees Celsius | 30 seconds |
| 48 degrees Celsius | 30 seconds |
| 68 degrees Celsius | 120 seconds |
| Repeat 34 times | - |
| 68 degrees Celsius | 300 seconds |
| Hold at 12 degrees Celsius until collected from thermocycler | |

**Running Gels:** The samples were retrieved from the thermocycler and run on a 1% agarose gel in TBE (54 g Tris base, 27 g boric acid, 20 ml 0.5M ethylenediaminetetraacetic acid, 1 L $H_2O$) to visualize PCR products. Successful samples were those that appeared as a single band between positions 27 and 1492 of bacterial 16S rRNA. A random sample of 30 successful PCR products from each sterilization method were sent for DNA sequencing.

**Bacterial Identification:** When the results of the DNA sequencing returned, each sequence was input into https://blast.ncbi.nlm.nih.gov/Blast.cgi database to determine the closest relative. The identified bacterial species were recorded by sterilization method and compared.

2.3: Comparison of Two Sterilization Techniques on Known Pathogens

**Animals** - Pig skin (Sus scrofa domesticus) from a local slaughterhouse was collected and stored at 4 degrees Celsius in the refrigerator for 24 hours. It was then transferred to the freezer at -18 degrees Celsius until thawed for use in the study.

**Sample Collection -** Once thawed, the skin was cut into sections at least 4 x 4 inches in size. Each section of skin was then stretched tightly and pinned to a dissection tray to be held steady. The skin was shaved using a 10 blade across the 4 x 4 inch section. The skin was rinsed under sterile distilled water to remove any loose hair. It was then sterilized for 5 minutes using 70% isopropyl alcohol wipes. Four quadrants were then drawn on the skin with a permanent marker to indicate the four collection areas. This can be seen in Figure 6 below.

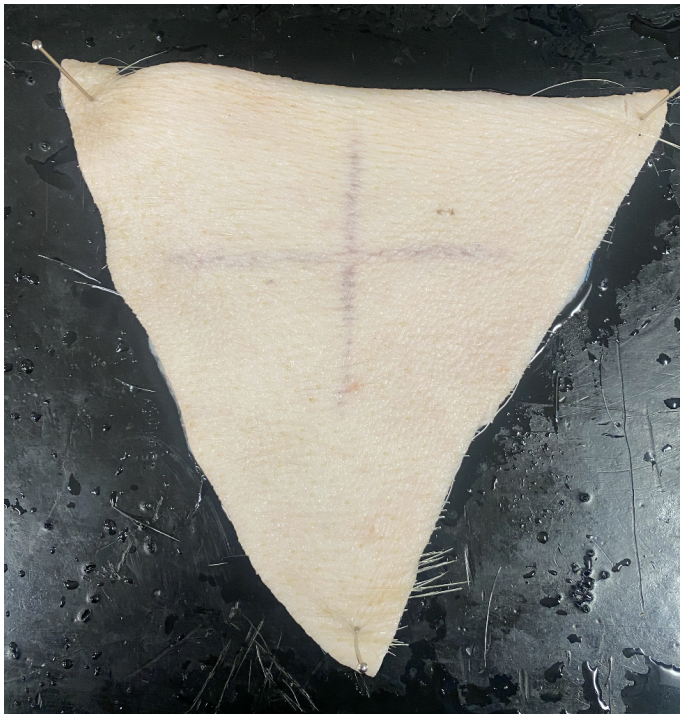

Figure 6: Pig skin section pinned to the dissection board with sharpie indicating the sites of collection

Following sterilization, the quadrants were swabbed and streaked following the previous pattern on Muller Hinton Agar plates, and then incubated for 24 hours at 37 degrees Celsius to provide a control sample before testing. A 6-inch long dry cotton sterile swab was used to collect control samples. The sterile swab was swiped across the shaved area 10 times back and forth starting from the top left corner to the bottom left corner of the area with the head of the swab directly on the skin. The swabbing pattern used was the same from the previous protocol in section 2.2.

400 microleters of overnight liquid culture of *Staphylococcus epidermidis* (safe relative of MRSA) suspended in LB were pipetted into each quadrant. Approximately $3.2 \times 10^8$ bacteria were applied to each 2 square inch section of pig skin. The liquid culture was spread evenly over

the area using a plate spreader, and allowed to dry for five-minutes. The quadrants were then swabbed again and plated to provide another control. The two sterilization protocols used in section 2.1 were then completed on opposite sides of the medial plane of the quadrants using generic brand materials, and the areas were streaked and plated as described in section 2.1 but only after the full five minutes of sterilization. This was then repeated 10 trials, and another 10 trials using the same number of *Pseudomonas aeruginosa putida* (safe relative of *Pseudomonas aeruginosa*) as the bacteria.

**Quantification of Colony Forming Units -** Following incubation, the number of colonies on the plates were counted for each sample and recorded. If there were colonies found on the plate from the pre-sterilization, this number was subtracted from the number of colonies counted from the sterilization trials to make sure that only the known bacteria were being included in counts.

**Statistical Analysis -** A matched pair sample t-test was performed to see if there was a difference between the two means of colony forming units from the two separate protocols. There were 20 samples from each sterilization method, and 10 for each type of bacteria. The equation can be seen above in section 2.2.

# 3: Results and Analysis

3.1: Comparison of Two Sterilization Techniques in Equine Patients

Table 6: Mean colony count per method including standard error of the mean for all collection areas combined.

| Method | Control | 30 seconds | 1 minute | 5 minutes |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | Too many to count | 13. ± 4.0 | 11. ± 3.9 | 5.1 ± 1.4 |
| Rounds of 0.75% povidone iodine and 70% isopropyl alcohol | Too many to count | 18 ± 4.6 | 11 ± 3.1 | 8.0 ± 3.9 |

The paired t-test was run to compare the means after the full five minutes of sterilization. The t-value was 1.7, resulting in a p-value of 0.10. A p-value less than 0.05 was needed in order to reject the hypothesis. This indicates 5 minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol is equally effective in reducing colony forming units as rounds of 0.75% povidone iodine and 70% isopropyl alcohol on all collection areas.

Table 7: Mean colony count per method including standard error of the mean for samples collected from the hawk region.

| Method | Control | 30 seconds | 1 minute | 5 minutes |
|---|---|---|---|---|
| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | Too many to count | 7.5 ± 3.9 | 4.2 ± 3.3 | 3.2 ±1.6 |
| Rounds of 0.75% povidone iodine and 70% isopropyl alcohol | Too many to count | 16. ± 14. | 6.8 ± 3.8 | 4.2 ± 3.9 |

The t-value was -0.48, resulting in a p-value of 0.65. A p-value less than 0.05 was needed in order to reject the hypothesis. This indicates that 5 minutes of 0.75% povidone iodine

followed by 70% isopropyl alcohol is equally effective in reducing colony forming units as rounds of 0.75% povidone iodine and 70% isopropyl alcohol when used on the hawk region.

Table 8: Mean colony count per method including standard error of the mean for samples collected from the carpus region.

| Method | Control | 30 seconds | 1 minute | 5 minutes |
|---|---|---|---|---|
| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | Too many to count | 6.5 ± 5.2 | 4.2 ± 3.9 | 2.0 ± 2.0 |
| Rounds of 0.75% povidone iodine and 70% isopropyl alcohol | Too many to count | 9.5 ± 8.4 | 5.2 ± 4.4 | 2.8 ± 2.0 |

The t-value was -0.73, resulting in a p-value of 0.50. A p-value less than 0.05 was needed in order to reject the hypothesis. This indicates that 5 minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol is equally effective in reducing colony forming units as rounds of 0.75% povidone iodine and 70% isopropyl alcohol when used on the carpus region.

Table 9: Mean colony count per method including standard error of the mean for samples collected from the palmer digital region.

| Method | Control | 30 seconds | 1 minute | 5 minutes |
|---|---|---|---|---|
| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | Too many to count | 17. ± 11 | 11. ± 10. | 5.7 ± 4.3 |
| Rounds of | Too many to | 20. ± 12. | 14 ± 11. | 8.0 ± 9.6 |

| | | | | |
|---|---|---|---|---|
| 0.75% povidone iodine and 70% isopropyl alcohol | count | | | |

The t-value was -0.67, resulting in a p-value of 0.53. A p-value less than 0.05 was needed in order to reject the hypothesis. This indicates that 5 minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol is equally effective in reducing colony forming units as rounds of 0.75% povidone iodine and 70% isopropyl alcohol when used on the palmar digital region.

Table 10: Mean colony count per method including standard error of the mean for samples collected from the midline region.

| Method | Control | 30 seconds | 1 minute | 5 minutes |
|---|---|---|---|---|
| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | Too many to count | 17. ± 13. | 18. ± 7.8 | 8.3 ± 2.4 |
| Rounds of 0.75% povidone iodine and 70% isopropyl alcohol | Too many to count | 21. ± 5.0 | 15. ± 6.4 | 10. ± 7.8 |

The t-value was -0.47, resulting in a p-value of 0.66. A p-value less than 0.05 was needed in order to reject the hypothesis. This indicates that 5 minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol is equally effective in reducing colony forming units as rounds of 0.75% povidone iodine and 70% isopropyl alcohol when used on the midline region.

Table 11: Mean colony count per method including standard error of the mean from samples collected from the sacroiliac region.

| Method | Control | 30 seconds | 1 minute | 5 minutes |
|---|---|---|---|---|
| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | Too many to count | 15. ±7.7 | 17. ± 11. | 6.3 ±2.0 |
| Rounds of 0.75% povidone iodine and 70% isopropyl alcohol | Too many to count | 22. ± 9.6 | 13. ± 4.9 | 15. ± 14. |

The T-value was -1.4, resulting in a p-value of 0.23. A p-value less than 0.05 was needed in order to reject the hypothesis. This indicates that 5 minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol is equally effective in reducing colony forming units as rounds of 0.75% povidone iodine and 70% isopropyl alcohol when used on the sacroiliac region.

3.2: PCR Results

Though 60 samples were sent for sequencing, only 27 were successfully sequenced. 10 samples were sequenced from 5 minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol, and 17 samples were sequenced from five minutes of rounds of 0.75% povidone iodine and 70% isopropyl alcohol.

Table 7: Bacteria with the highest percent identity to the query sequenced. Numbers in parenthesis indicate multiple samples sharing the same bacteria with the highest percent identity to the query sequenced.

| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | Rounds of 0.75% povidone iodine and 70% isopropyl alcohol |
|---|---|
| *Bacillus proteolyticus (3)* | *Bacillus proteolyticus (6)* |
| *Bacillus clarus (3)* | *Bacillus clarus (5)* |
| *Lysinibacillus capsici* | *Bacillus pseudomycoides* |
| *Metasolibacillus fluoroglycofenilyticus* | *Bacillus marcorestinctum* |
| *Acinetobacter johnsonii* | *Peribacillus acanthi* |
| *Psychrobacter halodurans* | *Alcaligenes ammonioxydans* |
| | *Gottschalkia purinilytica* |
| | *Micrococcus yunnanensis* |

The majority of bacteria sequenced from both methods were *Bacillus*. 5-minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol resulted in 80% *Bacillus* species. Rounds of 0.75% povidone iodine and 70% isopropyl alcohol resulted in 82% *Bacillus* species. *Bacillus* are gram-positive rod shaped bacteria. It is consistent with the primary literature that they were able to withstand sterilization, as they are resistant to antiseptics due to the presence of endospores. Endospores create a protective layer around the bacteria making them harder to eradicate through general sterilization procedures (Friedline, 2015).

3.3: Pig Skin Testing

The mean colony forming units for both bacteria after sterilization can be seen below.

Table 8: Mean colony count with margin of error found on the plates for both sterilization methods on two different known induced bacteria

| | *Pseudomonas Aeruginosa* | *Staphylococcus Epidermidis* |
|---|---|---|

| | | |
|---|---|---|
| 5 Minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol | 1.1 ± 1.0 | 0.20 ± 0.26 |
| Rounds of 0.75% povidone iodine and 70% isopropyl alcohol | 0 ± 0 | 0.40 ± 0.78 |

The means of both sterilization methods for both bacteria types were compared using a paired t-test. The results of the matched pair t-tests can be found in the table below.

Table 9: Matched pair t-test results for both sterilization methods on *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*

| *Pseudomonas aeruginosa* | *Staphylococcus epidermidis* |
|---|---|
| t = 2.2 | t = 0.45 |
| p = 0.06 | p = 0.66 |
| Not significant | Not significant |

There is no significant difference between the two sterilization method's colony reduction capacity on both *Pseudomonas aeruginosa* and *Staphylococcus epidermidis,* and the hypothesis is accepted.

# 4: Discussion, Conclusions, Recommendations

The goal of this project was to compare the two different pre-procedural sterilization protocols to determine if one was more effective at lowering colony forming units cultured from the sterilization site. The primary literature indicated minimal differences between the two antiseptics as discussed in Section 1.3, so the duration and order of usage should not affect overall bacterial growth. The hypothesis of this project was that there would be no significant difference in the effectiveness of the two sterilization procedures in reducing colony forming units. The hypothesis would be rejected due to the experimental outcomes below.

1. There was a significant difference in the mean number of bacterial colonies cultured from collection swabs taken from equine patients after five minutes of each sterilization protocol.
2. The bacteria colonies sequenced from each sterilization method were significantly different in species composition.
3. There was a significant difference in the mean number of colonies cultured from collection swabs taken from pig skin induced with *Staphylococcus epidermidis* (safe relative of MRSA) and *Pseudomonas aeruginosa putida* (safe relative of *Pseudomonas aeruginosa*) after five minutes of each sterilization protocol.

The hypothesis was accepted as there was no significant difference observed between the two sterilization protocols for any of the experiments. This indicates that 5 minutes of 0.75% povidone iodine followed by 70% isopropyl alcohol is equally effective in reducing colony forming units as rounds of 0.75% povidone iodine and 70% isopropyl alcohol on all collection areas. Additionally, we are 95% confident that there is no significant difference between the two

sterilization method's colony reduction capacity on both *Pseudomonas aeruginosa* and *Staphylococcus epidermidis,* and the hypothesis is accepted. Both methods had *Bacillus* bacteria with the highest percent identity to the query sequenced.

*Bacillus* species have endospores which protect the bacteria and make them resistant to regular sterilization protocols. The most common *Bacillus* pathogen affecting horses is *Bacillus anthracis* (Hugh-Jones, 2019). Though this pathogen is relatively uncommon in horses compared to *MRSA* or *Pseudomonas Aeruginosa,* it is still a risk. This pathogen causes anthrax, a fatal disease in horses that can also be spread to humans. The pathogen can enter through open cuts or sores, thus it can be contracted during surgery. The bacteria produces a toxin that leads to cell and tissue death. Symptoms include fever, severe colic, severe swelling, difficulty breathing, seizures, bloody stool, and death (Hugh-Jones, 2019). Luckily, *Bacillus anthracis* is susceptible to antibiotics.

As discussed in section 1.4, there are many ways to reduce *Bacillus* species. Many cannot be used on equine skin due to risk of tissue damage. *Bacillus* is susceptible to killing dry heat such as gamma radiation or UV exposure, as well as high temperatures or wet heat (Setlow, 2006). Bacillus can also be killed by other chemicals such as HCl, NaOH, $ClO^-$, and $H_2O_2$ (Setlow et al, 2002). These can be used to remove *Bacillus* from non-organic surfaces. Additionally, chlorine dioxide has been found to be effective in killing *Bacillus* species (Setlow et al, 2002). In a recent FDA approved trial, researchers compared activated chlorine dioxide and normal sterile saline as an antiseptic for wound irrigation on 193 human patients suffering from fresh lacerations. It was concluded that there were no significant differences in post suturing infections, adverse reactions, and cosmetic outcome between activated chlorine dioxide and normal sterile saline (Valente et al, 2014). Further research on the use of activated chlorine dioxide as an antiseptic pre-surgery on equine patients could be performed to determine if the

chemical would be effective at reducing the Bacillus species left over after the usage of 0.75% iodine povidone and 70% isopropyl alcohol.

There were confounding variables that could have affected the data outcome of this research and could be corrected if the study were repeated. For the first experiment, the horses were fully awake during the testing, and had varying temperaments. They often moved around, which could have induced more bacteria into the site during the trials. In the future if this experiment were to be repeated, it may be beneficial to test on horses already undergoing anesthesia to reduce risk of confounding movement. Additionally, a larger and more diverse pool of horses should be used in the future to confirm the results. For the second experiment, due to time and monetary restrictions, a random sample of 60 of the 394 bacterial colonies were sent for DNA sequencing, and only 27 were successfully sequenced. If repeated, more colonies should be sent out to see if there are other patterns in the types of bacteria that withstand both sterilization protocols. Finally, for the third experiment, pig skin was used as a replacement for equine skin. Pig skin is more porous and has a different hair texture, likely affecting the outcome of the experiment. In addition, even though the skin was pre-sterilized, it was not 100% sterile. For a future experiment, it would be beneficial to repeat the trials on equine skin to elicit more accurate results.

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