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# The transfer of commensal and transient bacteria into deep tissue using dry needling needles through agar media designed to mimic the elasticity of human skin

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# **The Transfer of Commensal and Transient Bacteria into Deep Tissue Using Dry Needling Needles Through Agar Media Designed to Mimic the Elasticity of Human Skin**

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Departmental Honors Thesis  
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## **DEDICATION**

To my father Wellyntong M. Bretón and my mother Maria A. Bretón Guerrero, who immigrated to the United States of American from Santo Domingo, Dominican Republic, so that their three children could have the quality of life they were not given.

Para mi padre Wellyntong M. Bretón y mi madre María A. Bretón Guerrero, quienes emigraron a los Estados Unidos de Santo Domingo, República Dominicana , para que sus tres hijos podrían tener la calidad de vida que ellos no recibieron.

## ABSTRACT

Needle insertion into skin for the purpose of alleviating muscle pain began with the emergence of traditional Chinese medicinal (TCM) acupuncture. “Dry needling”, a recent alternative to acupuncture, involves inserting a monofilament needle down into the deep tissue of a patient’s skin in order to reach the affected muscle. Despite its effectiveness and low risk of infection, dry needling remains questionable with respect to its safety and the lack of research to confirm the low risk of bacterial transmission via the dry needling needles. In this microbiological study, highly concentrated Tryptic Soy Agar was utilized to simulate the elasticity of human skin, and the transmission of commensal and transient bacterial species into “deep tissue” (agar) was analyzed. Transmitted cells of varying overnight culture dilutions were examined along the needle insertion in the agar and further analyzed at each depth of agar using a confocal microscope. Results showed that while *S. aureus* growth decreased along the length of the needle stab after an increase in the dilution of the culture, *E. coli* cells showed significant growth even in the highest dilution. This could be due to *E. coli*’s motile properties and their rod shape, which provides more surface area and thus more binding units that can facilitate adhesion to the needle. Confocal images of *E. coli* cells also demonstrated a high concentration of cells transmitted. The behavior exhibited by *E. coli* could suggest a higher risk of infection via dry needling than the potential risk of a *S. aureus* infection, however, the number of *E. coli* cells that were transmitted were still not enough to cause an infection in the tissue. This study serves to support the hypotheses made in a few other studies that the risk of infection from dry needling is tremendously low, and also showcases the behavior of *Staphylococcus aureus* and *Escherichia coli* in a highly elastic environment.

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*Dr. Randy Walker*

*Dacey Winkleman*

*Zac Cooper*

*Heather Harmon*

*Brittany Rock*

## BACKGROUND LITERATURE

Myofascia, sometimes referred to as “the tissue of movement”, is the dense tissue that covers the bones and muscles of the body (Center 2014). This tissue is not only tremendously tough, but it is also flexible, with a tensile strength of over 2,000 pounds (Center 2014). Musculoskeletal pain located in the myofascia is among the most common syndromes seen by family practitioners worldwide, accounting for the majority of acute and chronic pain complaints (Kalichman and Vulfsons 2010). This pain originates from a specific area of muscles or related fascia called myofascial trigger points (MTrP). A myofascial trigger point is a highly localized area of tense skeletal muscle fibers, which can be stimulated to produce two perceptible responses: acute or chronic pain in the affected muscle or a local twitch response (Kalichman and Vulfsons 2010). One example is the common patient complaint of muscle pain around the pericranial muscles, a syndrome clinically diagnosed as chronic tension headaches due to myofascial pain (Borg-Stein and Simons 2002). Several muscles that are most prone to myofascial tension include the *upper trapezius*, *scalene*, *sternocleidomastoid*, *levator scapulae*, and *quadratus lumborum*—muscles in the pelvic girdle, neck, and shoulders (Alvarez 2003). Refer to Figure 1 on p.654 of article “Trigger Points: diagnosis and pain management” by Alvarez JD and Rockwell PG found in American Family Physician to view the location of the most common myofascial trigger points. The muscles involved in these trigger points include the *latissimus dorsi*, *sternocleidomastoid*, *semispinalis capitis*, *infraspinatus*, *dorsal interossei*, *subclavis*, *sternalis*, *anterior deltoid*, *pectoralis major*, *palmaris longus*, *pectoralis minor*,

*pronator teres, sartoris, mcburney's point, gracilis, tibialis anterior, and the extensor digitorum longus.*

Epidemiological studies in the United States claim that MTrPs were the primary source of pain in 30-85% of patients treated in primary care or specialist pain clinic settings (Tough et al. 2009). Despite the numerous studies claiming that MTrPs are the leading cause of muscle pain affecting 10% of the adult population, musculoskeletal pain is frequently undiagnosed by physicians and physical therapists (Kalichman and Vulfsons 2010). Musculoskeletal pain that is not treated immediately can lead to chronic muscle pain that is not easily cured with non-invasive treatments such as massage, stretching, acupressure, ischemic compression, laser therapy, or transcutaneous nerve stimulation (Kalichman and Vulfsons 2010). No single strategy has proven to be universally successful (Kalichman and Vulfsons 2010). However, the modifying approaches to injection therapy have changed it into an effective method of alleviating chronic muscle pain in therapy facilities around the world. Physicians have been using acupuncture as a method of relieving chronic pain, and more recently, physical therapists have begun to practice dry needling as an alternative to acupuncture. Dry needling is a modification of acupuncture that can be better understood through the brief history of acupuncture and a look into its movement from Eastern to Western medicinal culture.

The practice of inserting needles into points of soft tissue tenderness was first introduced by Eastern Chinese traditional medicine about 2,000 years ago (Vickers and Zollman 1999). Although there were several Asian schools of thought regarding acupuncture, Chinese acupuncture dominated Eastern medicine, therefore in this

specific analysis the words “traditional” and “Chinese” may be used interchangeably and should be distinguished from Western, modern schools of thought. This process of needling is referred to as “acupuncture”, which etymologically originates from the Latin prefix *acus* meaning “needle” or “sharpness” and the English word *puncture* meaning the insertion of needles into a part for the production of counterirritation (Dorland 1915). Chinese acupuncturists practice with a specific philosophy of science that promotes human awareness and is based on qualitative assessments. According to Eastern traditional acupuncture, the human body is governed by an energy or force called the “qi”, which dictates the strength and quality of your muscles and organs. This qi circulates through the body through channels called meridians, which house the major organs of the body (Vickers and Zollman 1999). This philosophy is in compliance with Daoism, which is fundamentally described as an education in the fields of political and bodily cultivation that teaches a practice in the organization of state and body. According to Daoist philosophy, the flow of qi is essential to achieve a healthy, balanced life (Defoort 2007). Through the flow of qi, Chinese acupuncture targeted specific points along the twelve meridians that corresponded to the areas in which patients felt pain. They used fine, sterile, disposable needles for shallow insertion into the meridian points.

The transition of traditional Chinese acupuncture to the West was one that was slow and methodically questioned before the medical establishment in the United States accepted it. Acupuncture received widespread exposure in the U.S in the 1970’s, and upon its emergence was immediately scrutinized with skepticism. Westerners could not explain the obscure places where acupuncturists inserted the needles because they were

so distant from the areas of pain (Allchin 1996). They also quickly dispensed with the cosmological framework explained by the Chinese, replacing it with the Westernized pragmatic understanding of the natural world. In 1973, shortly after the advent of acupuncture in the United States, scientists found that many acupuncture points (71%) coincided with trigger points, or areas neurologist Henry Head—a renowned neurologist who broadened the scientific understanding of nerve ganglia, sensory spots, and peripheral nerve disorders—had declared “maximal tender areas” (Liao 1978; Swash 2008). After this discovery, Westerners have slowly begun to integrate similar acupuncture techniques that were practiced in the East, with a few differences in practice. In fact, there are now 40 accredited TCM (Traditional Chinese Medicine) acupuncture schools in the U.S (Sherman, Hogeboom, and Cherkin 2001). Westerners have taken the traditional method of utilizing fine, sterile needles to alleviate trigger points (acupuncture points) and rationalized the science behind it. Acupuncture in the context of the modern world can now be explained in relation to the nerve ganglia. The acupuncture mechanism involves three different pathways: the dorsal root of the spinal cord, the dorsal column nucleus of the medulla oblongata, and ventral posterolateral thalamic nucleus (Rong et al. 2011). Acupuncture creates an inhibitory effect on pain activity in the neurons; generally, the spinal dorsal horn plays the most important part in this inhibition (Rong et al. 2011). Through these pathways, acupuncture can target specific nerve ganglia to produce a pain relieving effect. This ideology that there was hard science behind needle insertion caused a paradigm shift in the way Western physicians viewed needle insertion. They were no longer basing their needling practice on the mystical concepts of Traditional Chinese Medicine, but created their own basis

of ideology based on concrete scientific explanations (Liao 1978). This led to the advent of dry needling—a new alternative to acupuncture that is based on a Western ideology of practice. Both of these needling practices are being utilized today, but there are potential risks of infection that should be considered.

While needling is effective for pain relief, inserting a foreign body into the skin creates an inherent risk for infection at the needling site. The National Health Institute states clearly in one of its patient handouts, “Since the skin is the body’s first defense against infection, it must be cleansed thoroughly before a needle is inserted”. Thus, any insertion into skin tissue requires a protocol for skin preparation such as disinfection. Research conducted as early as the 1960s by Dann and Koivisto & Felig with diabetic patients indicated that disinfection with alcohol is not necessary to prevent infection at needle insertion sites (Dann 1969; Koivisto and Felig 1978). While skin preparation does significantly reduce skin bacterial counts, further studies have shown there is no increased risk of infection if skin is not cleaned with alcohol (Council of Colleges 2014). The World Health organization affirmed this in 2003 by stating that if the skin is “clean” there is not a need to swab an insertion site regardless of the body area. The U.S Center for Disease Control and Prevention also reinforced this, stating that alcohol, soap, and water or chemical agents are not needed for preparation of the skin prior to vaccination, unless the skin is grossly contaminated or dirty (Modlinand 2001). While the majority of studies show that skin preparation prior to needle insertion is not necessary, many practitioners in healthcare facilities believe that the best practice guideline is to clean the skin to *reduce* the risk of infection even more (Mallett and Bailey 1996; Institute of Health 2012). The organisms that are most often responsible

for causing skin infections are *Staphylococcus aureus* and *Streptococcus pyogenes* (Council of Colleges 2014). Recently, infections caused by mycobacteria have posed a new threat due to their resistance to disinfectants such as 75% alcohol and chlorhexidine, an antiseptic antimicrobial agent (Fraser et al. 1992). The lack of knowledge towards acupuncture mycobacteriosis stems from the rare occurrence of these incidents and the research studies that have been done proving that skin can be adequately disinfected with alcohol (Woo et al. 2002). While there are numerous studies on the risk of infection in regards to acupuncture, there is insufficient research on the risks of infection in regards to dry needling. This is important because dry needling typically involves inserting the needle down into the deep tissue in order to reach the affected muscle, not shallow insertions as is most commonly used in acupuncture. The dry needling procedure involves multiple insertions of a monofilament, dry needle into MTrPs. (Audette and Bailey 2008; Bourne 2008). The name “dry” needling is derived from an older technique called trigger point injections. These injections were “wet”, meaning that they injected a liquid substance through a hypodermic needle. Dry needling uses a solid monofilament needle and does not inject anything and so it is “dry”.

Currently, the practice of dry needling by physical therapists is banned in ten U.S states, including Tennessee. Controversies concerning trigger point dry needling, commonly abbreviated as TDN, stem from lack of procedural techniques including proper protocol regarding skin preparation prior to insertion. The lack of knowledge about the risks for potential infection via dry needling has led to this inconclusive standard of sterilization in its protocol. Despite its effectiveness and presumed low risk

of infection, the practice of dry needling remains questionable with respect to its safety. Currently, there is no evidence that mandates the disinfection of skin prior to TDN, but there is still a theoretical risk for infection that must be accounted for (Dunning 2012).

## INTRODUCTION

During Fall 2014 UTC students Dacey Winkleman, Heather Harmon, Zac Cooper, and Brittany Rock of Dr. David Levine and Dr. Randy Walker (UTC's Physical Therapy Department), initiated a study of potentially infectious bacteria on subject skin in areas into which dry needles were inserted. Their study involved identification of bacteria found on the skin and removed from the skin surface upon removal of the needle from the skin. Specifically, their method was to compare the bacteria found on the skin surface prior to the needle insertion to the bacteria present on the needle after the insertion. In their study, 27 healthy individuals participated in a dry needling treatment performed by a licensed nurse practitioner (Winkleman 2014). The scope of their study consisted of 22 females and 5 males, ranging in age from 23-50 years. Volunteers were excluded from the study based on the following parameters: needle phobias, bleeding disorders, MRSA infection within the last year, immunocompromised, cold within the past week, or antibiotics within the past month. Furthermore, the participants were instructed to avoid showering within 12 hours of their scheduled dry needling treatment (Winkleman 2014). Five major trigger point locations were treated on each participant: *latissimus dorsi*, *sternocleidomastoid*, *semispinalis capitis*, *infraspinatus*, and *dorsal interossei* (foot). Prior to needling at each site, the skin was swabbed approximately 1-2 cm from the needling site. After the

experiment it was found that 100% of the patients had at least one needling site with existing bacteria. When incubation of the needles was complete, the samples were analyzed for varying levels of growth. For each needling site, the growth on the swab was significantly higher than the growth on the needle. The results of her study suggest that the potential for transfer of surface bacteria into the tissue is low. Thus, the study ultimately supported previous research claims that there is a relatively low to non-existent risk of infection in the practice of dry needling (Dann 1969). Although this study provided insight and implications on thoughts concerning the practice of dry needling, it also set the stage for further investigation and future research.

Dr. Spratt found it necessary to perform a more in-depth continuation of Dacey Winkleman's project. While Winkleman's project was efficiently executed, working with human subjects placed restrictions on the experimental design. By creating a more controlled microbiological environment, the ability to analyze the presence and depth of these bacteria in the skin could be more effective. This research experiment is limited to the microbiological aspects of Winkleman's study in that the results analyze and examine the bacteria transmitted more thoroughly. Additionally, since the initial study did not have any way to directly determine the transfer of skin-associated bacteria down into deep tissue (e.g., none of the subjects complained of infections at the needling sites on their bodies), we have no way of assessing whether and how much of the skin-associated bacteria might have been transferred deep into tissue. In an attempt to estimate potential transfer of bacteria from a surface mimicking skin into underlying tissue, in this study we created agar media designed to mimic the elastic modulus of human skin. Using Tryptic Soy Agar media, we were able to test potential transfer of

bacteria through an agar surface, and as deep into the media as the needles were pushed into the subjects. A *microbiological* analysis of bacterial transmission via the practice of dry needling will serve to establish a better-validated standard of the potential risk of infection. This is an issue that needs to be further studied due to the increasing popularity of dry needling as an alternative to acupuncture. With such a growing field of physical therapy and the application of dry needling in clinics, it is necessary to account for the potential numbers of bacteria introduced *deep* within the skin tissue. The purpose of this study was to create a controlled experimental study in which we used highly concentrated agar in order to thoroughly examine the process of bacterial transport into deep tissue via the dry needling needles. This study will provide tangible, empirical data that might impact the field of physical therapy, as it has been a controversial practice due to its lack of procedural protocol related to proper skin preparation prior to practice.

## **EXPERIMENTAL DESIGN**

### **Bacterial Species**

In this study, two categories of bacteria were utilized: commensal and transient. Commensal bacteria can be defined as bacteria that are typically part of the human flora (Tlaskalová-Hogenová, Štěpánková, and Hudcovic 2004). These bacteria can be specific to certain locations such as hair follicles, sweat glands, or any naturally warm, moist areas of the skin. The species of commensal bacteria associated with human skin that was utilized is *Staphylococcus aureus*. *S. aureus* is a major cause of skin, soft tissue,

respiratory, bone, joint, and endovascular infections, and it is one of the most common gram-positive pathogens that may cause sepsis (Lowy 1998). *S. aureus* is responsible for 46% of complicated skin infections (Rennie, Jones, and Mutnik 2003). There is one case report in which an acupuncture patient developed septicemia after the attending physician admitted to not properly preparing the skin prior to needle insertion (Simmons 2006). The other species of bacteria, not normally associated with human skin (transient species to the skin) was *Escherichia coli*. Transient bacteria are generally more amenable to transmission because they are more easily removed from the skin surface (Kohn et al. 2003). *E. coli* was selected because it is one of the most common gram-negative bacteria known for causing complicated skin and skin structure infections (Giordano et al. 2007), and because of its potential for contamination of skin via contact with fecal matter. *E. coli* is responsible for 7% of complicated skin infections (Rennie, Jones, and Mutnik 2003).

### **Preparation of Agar System**

The first step of the project involved developing multiple agar systems to mimic an elastic, skin-like environment. Tryptic Soy Broth (TSB) with different concentrations of Tryptic Soy Agar (TSA) was used to develop the prototype agar systems with varying elastic conditions. Screw thread test tubes in the size 10ml/10mm (volume/diameter) were utilized for agar preparation. It was necessary to determine how efficiently this agar displayed the presence of *S. aureus* at depth in the agar, before further investigation was performed with *E. coli*. This initial test was solely to detect the presence or absence of the cells beneath the agar surface, so the culture was not diluted. SEIRIN® J-Type disposable

acupuncture needles in size 8/0.30mm x 50mm (gauge/diameter x body length) were used for each insertion into the agar. Each SEIRIN® needle is sterilized with Ethylene-Oxide Gas (ETO) in compliance with World Health Organization Guidelines (SEIRIN-AMERICA 2008). The TSA medium clearly displayed the depth of the stab, demonstrating the parameters of significant growth along the needle stab. Once it was determined that there was visible growth along the needle, this experiment was replicated in September 2015—this time taking measurements of the depth of significant growth along the needle stab.

**Table 1:** Absorbance readings of dilutions #1

<b>Dilution</b>	<b>Absorbance</b>
1:10	0.094
1:5	0.182
<b>1:2</b>	<b>0.460</b>

### **Experiment 1 – *Varying concentrations of agar***

The first experiment in September 2015 involved nutrient media of four different agar concentrations (0.5%, 1.5%, 3%, 5%) with the purpose of finding the agar system that most closely resembled the elasticity of human skin. Like the previous experiment, TSA was made using varying concentrations of granulated agar, transferred into 10 ml test tubes, and sterilized in the autoclave. For the first trial of agar systems, three replicates were made of each of the four concentrations for a total of twelve tubes. An overnight culture of *S. aureus* was used to create three dilutions (1:10, 1:5, and 1:2) in sterile saline

of the original culture. The dilution with an absorbance value closest to 0.5000 under an optical density of 600 nm was selected (**Table 1**), so that an appreciable amount of bacterial cells could be transferred. A Fisher Scientific 415 Spectro Master spectrophotometer was utilized to determine the absorbance of each dilution. A 5-microliter drop of the 1:2 dilution was added to each tube at the top center and allowed to air dry for 15 minutes. SEIRIN® acupuncture needles were then used to stab down through the dry drop of cells into the agar as deep as possible. After this procedure was done for all 12 tubes, they were incubated for 48 hours at 37 degrees Celsius. Measurements of the maximal depth of significant growth of the bacteria as a percentage of the total depth of the stab were made.

$$\textit{Percentage Growth} = \frac{\textit{Total depth of stab}}{\textit{Total depth of significant growth}} \times 100$$

After attempting several methods of transferring the sterile agar into the test tubes, it became evident that the most effective and sterile method of transferring the 5% concentrated agar was to use 5-ml pipets with sterile tips to manually transfer the agar into the test tubes and then sterilize them in the autoclave. This method worked well with the 5% agar, but was ineffective when we increased the concentration of agar to a thickness of 10%. The high concentration of the agar prevented it from dissolving clearly in the medium. A slow increase in temperature was then done to dissolve the agar more efficiently, however, this caused the agar to burn and stick to the bottom of the flask. Similar complications were experienced when the concentration of the agar was decreased from 10% to 7.5% due to its thickness. The 7.5% agar was pipetted

successfully, however, contamination was present in all of the tubes after incubation. It was anticipated that mimicking the actual elasticity of skin would be an issue, therefore the experiment continued with the closest possible agar system to achieving the ideal skin conditions.

## **Experiment 2 – Varying dilutions of overnight cultures**

**Table 2:** Absorbance readings of dilutions #2

<b>Dilution</b>	<b>Absorbance</b>
1:1	0.738
1:2	0.439
1:10	0.085
1:25	0.035
1:50	0.017
1:100	0.008

For the next experiment in October 2015, the concentration of agar was kept constant at 5% and the dilution of the culture was varied. 5% concentrated media was used for all 3 replicates of the five dilutions (1:2, 1:10, 1:25, 1:50, 1:100) of overnight *S. aureus* culture for a total of 15 tubes. The absorbance of each dilution was recorded under a 600 nm optical density (**Table 2**). The inoculation procedure was similar to the September 2015 experiment with a 5-microliter drop of overnight *S. aureus* culture added to the top center of each tube and a stab through the dry drop of cells as deep as possible. The tubes were then incubated for 48 hours at 37 degrees Celsius. Measurements of the maximal depth of significant growth of the bacteria as a percentage of the total depth of the stab were made. This experiment was replicated with cultures of a higher dilution, in order to determine the dilution that would result in the lowest transmission of bacterial cells. This exact

procedure was performed of pushing the cells down and taking measurements of the maximal depth of growth as a percentage of the total depth of the stab—this time with cultures diluted to 1:100, 1:150, and 1:200.

**Table 3:** Absorbance readings of dilutions #3

Dilution	Absorbance
1:100	0.010
1:150	0.004
1:200	0.002

After completing these tests with *S. aureus*, the same procedure was performed with *E. coli* using dilutions of 1:25, 1:50, 1:100, 1:150, and 1:200 (**Table 3**).

### **Experiment 3 – Viable plate counts**

The next phase of the study was to serially dilute the mixtures and streak them onto plates, incubate these plates, and then do a viable plate count to enumerate the amount of cells in each original culture (Hartsock 2004; Academy 2016). This was done with both *S. aureus* and *E. coli* overnight cultures. A viable plate count for each culture determines the approximate the number of colony forming units in each overnight stock solution. The CFU/mL (colony-forming units per milliliter) of the original cultures were calculated using the number of colonies divided by the total dilution factors of the plates.

$$CFU/mL \text{ in original culture} = \# \text{ of colonies} / TDF$$

Following the viable plate count, it was necessary to visualize these cells using the confocal microscope. The reporter gene used to fluorescently label the *E. coli* cells, could not be used to transform *S. aureus* because of its unique metabolism. In general, most strains of *S. aureus* are impermeable to the uptake of foreign DNA due to certain restriction enzymes in their genome (Monk et al. 2012). A type III restriction endonuclease prevents horizontal gene transfer in wild-type strains of *S. aureus*, and cuts up any foreign DNA that attempts to invade the cell (Corvaglia and François 2010). Therefore, only *E. coli* was examined using the confocal microscope.

## **Fluorescence Microscopy**

An Olympus Fluoview FV1000 confocal microscope was used to detect the presence of bacteria along the needle insertion site at each depth of agar (1cm, 2.5 cm, 4cm, and 5cm). Confocal microscopes are useful for examining the trafficking of fluorescent-labeled molecules and marking of specific live cells using multiple laser light scanners (World-leading Optics 2012). GFP (Green Fluorescent Protein), a protein produced by the jellyfish *Aequorea victoria*, was used to detect the cells under the confocal microscope. GFP is a unique reporter gene due to its independence or lack of a requirement for substrates or cofactors (Cowan et al. 2000). It can serve as a tagging molecule when it is excited by blue UV light and emits green fluorescence. This study required the use of a pGLO plasmid—an engineered plasmid that is used as a vector to induce the expression of a particular gene in a bacterial cell. The plasmid contains the reporter gene GFP as well as an ampicillin resistance gene. A pGLO plasmid was injected into the gene of protein kinase c in *E. coli*, resulting in the expression of the GFP

gene in the bacterial cell (Blaber 2004). A UV light was aimed at the cells and the fluorescence associated with GFP was observed at the location of each bacterial cell. Prior to use of the confocal microscope, a special agar system was prepared with the antibiotic ampicillin (normally *E. coli* cannot survive in its presence) and arabinose sugar. The *E. coli* cells grew in this ampicillin-containing agar, validating that the *E. coli* was successfully transformed with the pGLO plasmid that included an ampicillin resistant gene. Arabinose stimulates RNA polymerase to produce GFP in the transformed *E. coli*, thus it “turns on” the gene that codes for GFP. The cells glowed, revealing that RNA polymerase, whose activation was induced by arabinose, had transcribed the gene for GFP. Dr. Margaret Kovach provided transformed *E. coli* cells that were already introduced to pGLO; therefore the only requirement was to prepare the “amp/arab” (ampicillin/arabinose) containing media in order to visualize the transformed cells. Ampicillin and L-arabinose are heat sensitive, thus they were filter sterilized prior to their addition to the agar.

The final procedure involved the preparation of amp/arab containing agar so that we could visualize the fluorescent-labeled *E. coli* cells. The agar was prepared in 10ml plastic test tubes to facilitate the cutting of the agar into sections for analysis of the bacteria at each depth. A drop of cells from each dilution was placed at the top center of the agar tubes, as was done with the previous needling experiments. The needles were inserted through the drop of cells, but following this procedure it was necessary to immediately cut the plastic test tubes at their closed ends, push up the agar through each tube, and cut up the agar producing a 1mm sample (about the thickness of a dime) taking

from different depths. The samples of agar were placed on a microscope slide and viewed under the confocal microscope.

All procedures in these experiments were performed in compliance with aseptic laboratory techniques and to a standard that prevented the contamination of anything or anyone in the vicinity of these experiments.

### **Statistical Analysis:**

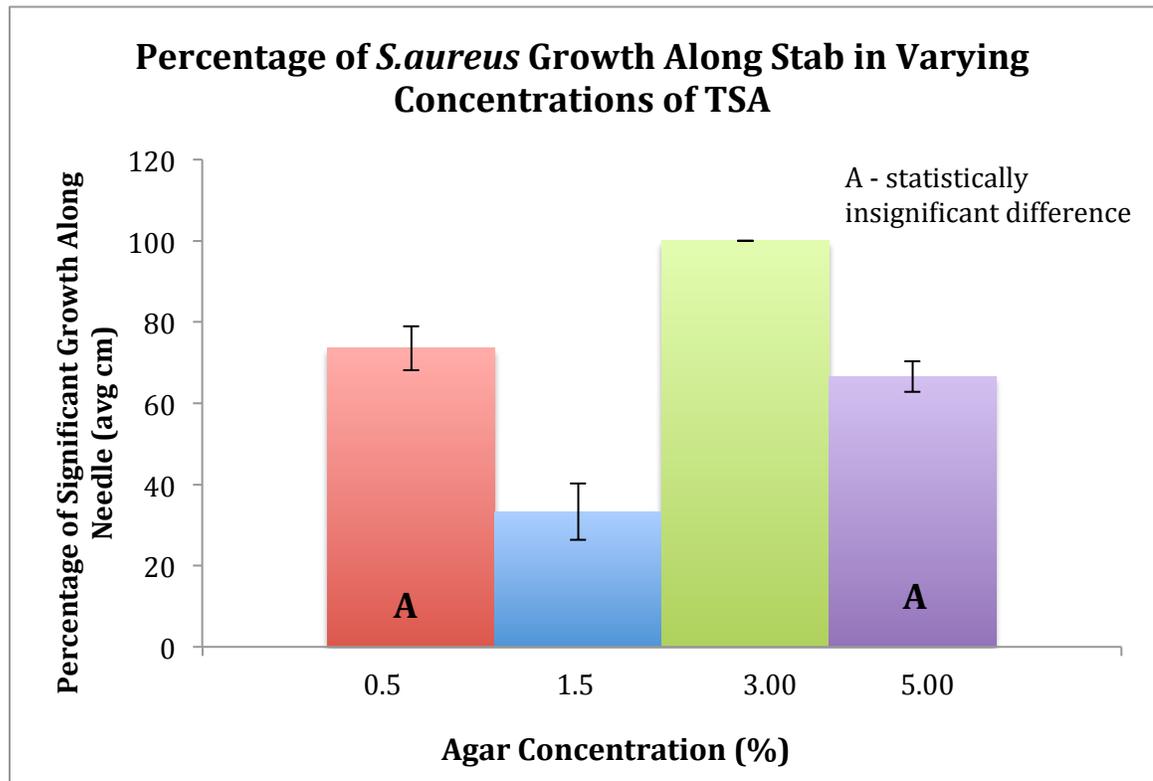
The statistical test used to verify significant trends in the results of this study was a student t-test under a significant P value of  $< 0.05$ . A student t-test statistical analysis is useful for small samples such as those used in this study. It involves finding a calculated mean, a standard deviation ( $\sigma$ ), and a standard error for each data set. A two-tailed t-test was then run on each data set and P values for each series of data were compared. P values  $< 0.05$  were considered to be statistically significant while values  $> 0.05$  were not statistically significant.

## **RESULTS**

### **Experiment 1 – *Varying concentrations of agar***

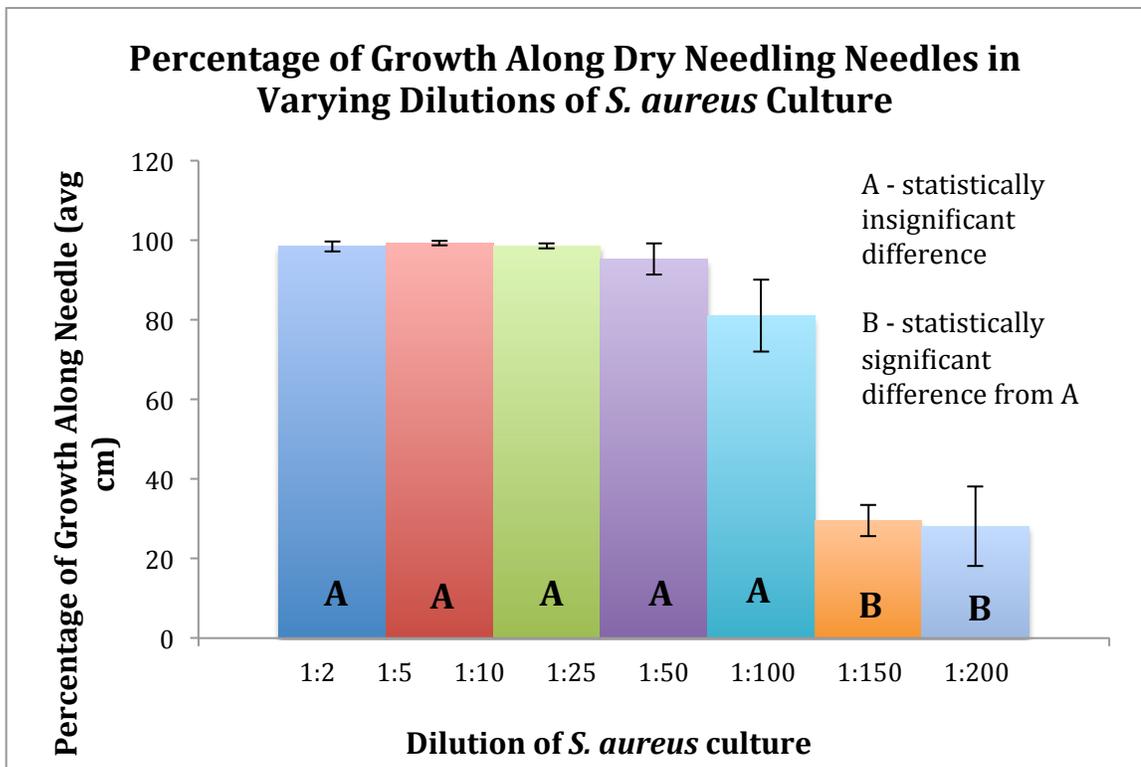
After conducting each experiment, the following results were obtained. The results of the September 2015 experiment revealed the 5% agar concentration to be the most suitable for the continuation of the project. Measurements of the significant growth of *S. aureus* along the stab were taken as a percentage of the total depth of the stab. This was done for all replicates A, B, and C and an average percentage was calculated for each concentration. The graph in **Figure 1** demonstrates the results for each agar concentration

along with their respective standard error. There was no significant pattern with the amount of bacterial growth and the concentration of agar. The purpose of this test was to determine the elasticity of 5% concentrated agar compared to that of skin. A number of studies have been done to determine the force needed to penetrate mammalian skin by a needle, but none of these studies have provided conclusive data to validate the penetration models (Shergold and Fleck 2005). Nonetheless, broad conclusions can be drawn from the data in those studies. These studies revealed that the force needed to penetrate the skin varied depending on the area of the skin being penetrated. For instance, the force needed to penetrate a muscle or skin tissue was much higher than that of fat tissue. Similarly, the penetration force also depends on the pre-stretch of the skin, the velocity of the penetrator (needle), the age of the person, and the moisture of the



**Figure 1:** Percentage of growth in varying agar concentrations

skin (Shergold and Fleck 2005). The mechanical properties of skin are highly dependent on these factors; therefore there is no single value that provides the elasticity or tensile strength of human skin. One particular study calculated the average elastic modulus—the ratio of force exerted upon a substance to the resultant deformation—of human skin to be 98.97 MPa (megapascals), which converts to 98970 kNm<sup>-2</sup> (Gallagher, Annaidh, and Bruyère 2012). The elastic modulus of a 3% agar gel is 700 kNm<sup>-2</sup> (kilonewton per meter squared) (Ross 1999). With only a two percent increase, it can be assumed that 5% concentrated agar is comparable to 3% concentrated agar. 700 kNm<sup>-2</sup> converts to 0.7 MPa compared to the skin’s modulus of 98.97 MPa. Due to the limitations of UTC’s microbiology laboratory, and the specific tools that were lacking to achieve an elastic modulus of this magnitude, the project continued with the agar concentration closest to the elasticity of skin, which was the 5% concentrated agar.

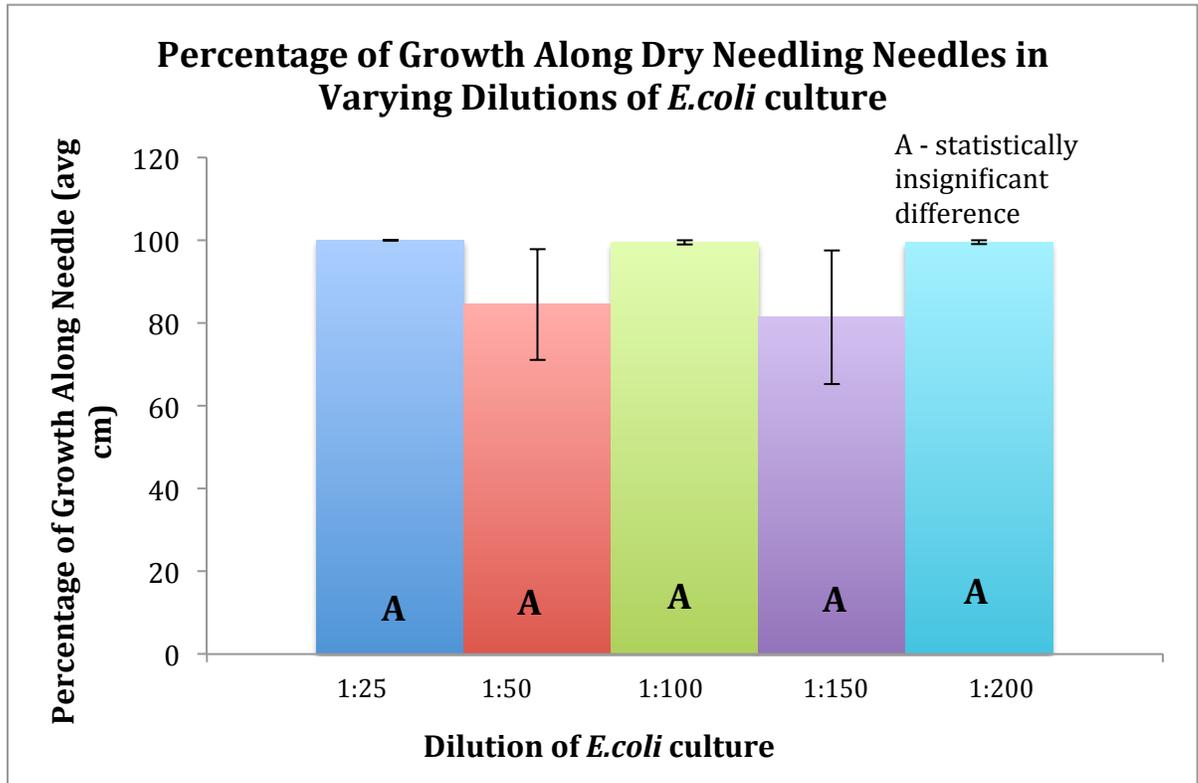


**Figure 2:** Percentage of *S. aureus* growth along needle insertion

## Experiment 2 – Varying dilutions of overnight cultures

Next, the results of the varying dilutions using the fixed 5% agar were documented.

**Figure 2** demonstrates the average percentages of growth along the needle for each dilution of overnight *S. aureus* culture and their respective standard error.



**Figure 3:** Percentage of *E. coli* growth along needle insertion

The data from dilutions 1:2, 1:10, 1:25, 1:50, and 1:100 did not reach statistical significance after running a  $P < 0.05$  student t-test. However, these dilutions all proved to be significantly different from both dilutions 1:150 and 1:200 with  $P$  values  $< 0.05$ , representing a significant variance in the amount of *S. aureus* cells that were transmitted deep into the agar as the dilution increases. In dilutions 1:150 and 1:200, the percentage of growth along the needle drops down to less than forty percent. A similar graph was generated with the varying dilutions of *E. coli* and is shown in **Figure 3**. Contrary to the

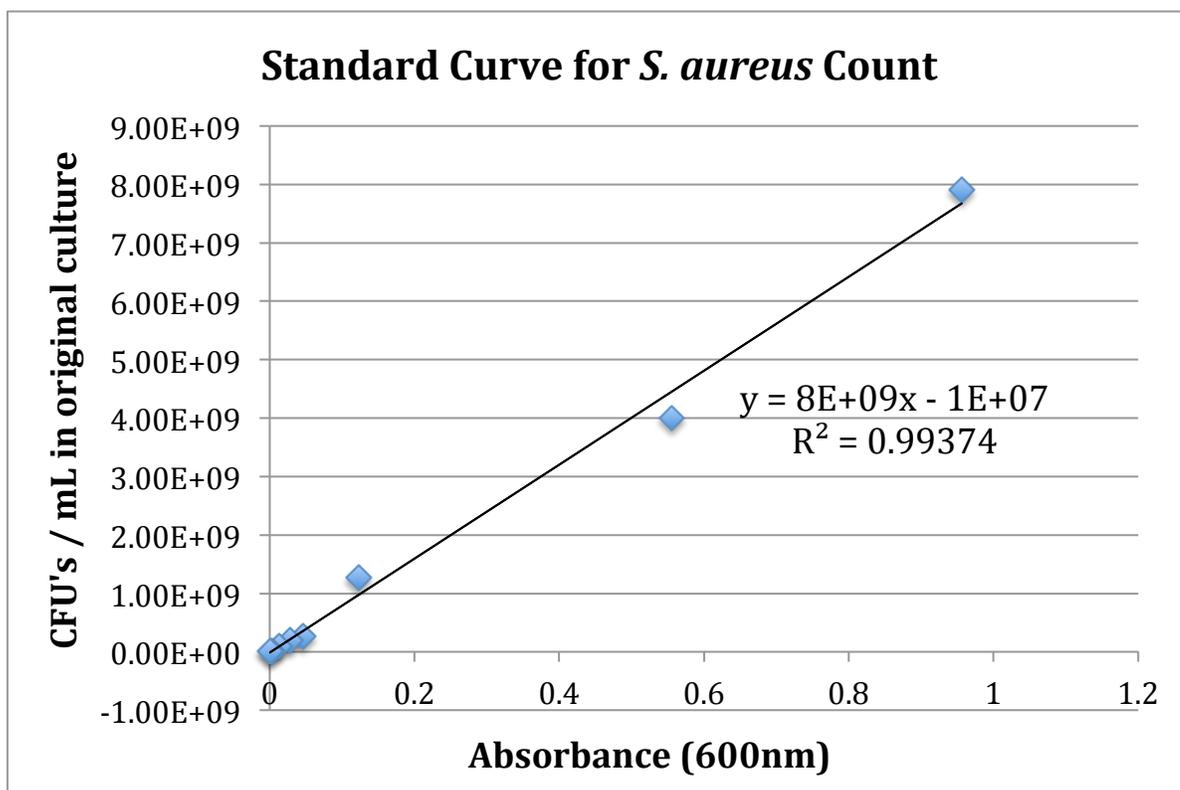
*S. aureus* culture, the *E. coli* cells did not vary depending on the dilution of the culture. The difference between the data of each dilution did not reach statistical significance and all yielded a P value > 0.05.

### Experiment 3 – Viable plate counts

After conducting a viable plate count, an estimation of the number of bacterial cells transferred via the needle was calculated. A sample calculation scheme using the 1:1 dilution of *S. aureus* is shown in **Appendix 1**.

**Table 4:** *S. aureus* enumeration data

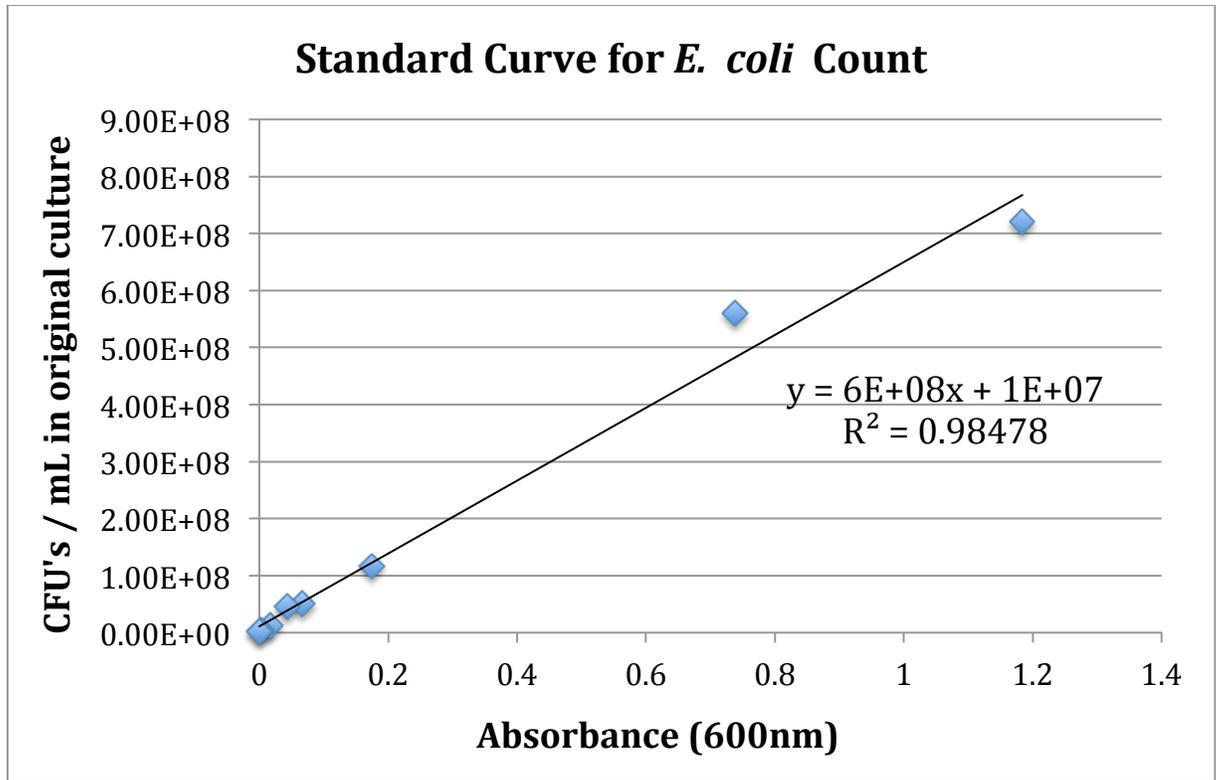
<b><i>S. aureus</i> Overnight Culture CFU</b>							
<b>Dilution</b>	<b>Absorbance</b>	<b>TDF</b>	<b># of colonies</b>	<b>CFU/mL</b>	<b>CFU/μL</b>	<b>CFU in 5μL</b>	<b>CFU via needle</b>
1:1	0.956	10 <sup>-8</sup>	79	7.90E+09	7.90E+06	3.95E+07	1.42E+05
1:2	0.555	10 <sup>-8</sup>	40	4.00E+09	4.00E+06	2.00E+07	7.20E+04
1:10	0.122	10 <sup>-7</sup>	127	1.27E+09	1.27E+06	6.35E+06	2.29E+04
1:25	0.046	10 <sup>-6</sup>	266	2.66E+08	2.66E+05	1.33E+06	4.79E+03
1:50	0.028	10 <sup>-6</sup>	195	1.95E+08	1.95E+05	9.75E+05	3.51E+03
1:100	0.012	10 <sup>-6</sup>	106	1.06E+08	1.06E+05	5.30E+05	1.91E+03
1:500	0.003	10 <sup>-4</sup>	178	1.78E+06	1.78E+03	8.90E+03	3.20E+01
1:1000	0	10 <sup>-4</sup>	97	9.70E+05	9.70E+02	4.85E+03	1.75E+01



**Figure 4:** *S. aureus* bacterial enumeration curve

**Table 5:** *E. coli* enumeration data

<b><i>E. coli</i> Overnight Culture CFU</b>							
Dilution	Absorbance	TDF	# of colonies	CFU/mL	CFU/ $\mu$ L	CFU in 5 $\mu$ L	CFU via needle
1:1	1.184	10 <sup>-7</sup>	72	7.20E+08	7.20E+05	3.60E+06	1.30E+04
1:2	0.738	10 <sup>-7</sup>	56	5.60E+08	5.60E+05	2.80E+06	1.01E+04
1:10	0.175	10 <sup>-6</sup>	117	1.17E+08	1.17E+05	5.85E+05	2.11E+03
1:25	0.067	10 <sup>-6</sup>	51	5.10E+07	5.10E+04	2.55E+05	9.18E+02
1:50	0.043	10 <sup>-6</sup>	45	4.50E+07	4.50E+04	2.25E+05	8.10E+02
1:100	0.017	10 <sup>-5</sup>	124	1.24E+07	1.24E+04	6.20E+04	2.23E+02
1:500	0.003	10 <sup>-5</sup>	39	3.90E+06	3.90E+03	1.95E+04	7.02E+01
1:1000	0	10 <sup>-4</sup>	150	1.50E+06	1.50E+03	7.50E+03	2.70E+01



**Figure 5:** *E. coli* bacterial enumeration curve

Following the viable plate count it was necessary to generate a standard curve for each of the bacteria by plotting the known concentrations (CFU/mL) with their corresponding absorbance values. Cultures of an unknown concentration can now be plotted against these standard values and their concentrations can be determined. This is calculated using the equation on the graphs and plugging in the absorbance value for X and the solving for Y.

### **Fluorescence Microscopy**

The confocal microscope was used to view each dilution of *E. coli* at each depth of the needle stab. While the point of needle insertion was successfully found at each depth, a few of the agar slices were too thin or contained bubble indentations, making it difficult

to distinguish the needle hole from the other holes in the agar. The images included in this analysis only include those dilutions and depths for which the needle insertion point was localized. The diameters were taken of holes at each depth, and they were approximately equal to the needle diameter of 300 micrometers or 0.30mm. Several of the holes showed an extension of that diameter in a particular direction. It is likely that the agar tore slightly further than the diameter of the needle during insertion. All confocal images referred to in this analysis are found in **Appendices 5 and 6**. In the 1:25 dilution (Appendix 6A), there are innumerable cells seen at the 1 cm depth with areas of higher fluorescence intensity than others. Cells also appear to be aggregated on several locations around the needle stab in brighter areas. This 1:25 dilution displayed the highest amount of fluorescence, as is expected with a less diluted culture. Appendix 6B shows a comparison of the agar at a site away from the needle site with an increasingly luminescent area that is nearer to the insertion site. Appendix 6C shows a 1:50 dilution at 2.5 cm depth with an aggregation of cells along only one side of the needling site as one side is much more luminescent than the other. A sample taken from a 4 cm depth (Appendix 6D) shows a large number of bacteria, however this time little to no aggregation is seen. As the cultures continue to increase in dilution, there is a decrease in the amount of fluorescence seen along the needle stab. The most diluted culture was observed at 5 cm under a 600 X objective lens, where the individual *E. coli* cells can be seen more clearly. These results suggest that although *E. coli* cells seemed to grow along the entire needle stab even up to a 1:200 dilution (**Figure 3**), the amount of cells that reach the end of the needle for each dilution does, in fact, decrease as the dilution increases.

## DISCUSSION

After critically examining the findings of this study, several conclusions were made. The viable plate count results suggest that for both *E. coli* and *S. aureus*, the number of cells that are transferred deep into the “tissue” should consistently decrease as the dilution increases. However, contrary to that hypothesis, the results from the 5% agar needle insertions demonstrated that the significant growth along the needle insertions varied between the two cultures. While *S. aureus* showed a decrease in significant growth along the needle insertion in dilutions 1:150 and 1:200, *E. coli* showed no significant variance in the amount of significant growth. An explanation for this phenomenon could be the motile properties of *E. coli*. *E. coli* cells possess several extracellular helical thread-like structures called flagella that improve cell transport and sensory reception. Each *E. coli* flagellum has a rotary motor at its base that turns in a counterclockwise motion to facilitate the forward propulsion of these bacterial cells (Kearns 2010). While there are many studies focusing on the movement of bacterial cells in liquid mediums, much less is understood about how some bacteria use flagella to facilitate movement through solid mediums. Several studies discussing *E. coli* motility state that due to the multiple flagella found on the cell surface of *E. coli* cells, these bacteria can more easily move in unison through a solid medium with more propulsion force and at a higher speed than normal (Kearns 2010). This mechanism combining the properties of aggregation and movement across a solid medium is defined as “bacterial swarming” (Kearns 2010). It is also possible that the formation of a relatively large hole in the medium created space for water through which cells could swim. It is important to consider *E. coli*'s hyper flagellated morphology when studying their successful movement through a highly

elastic, thick, concentrated agar medium. This mechanism could permit *E. coli* cells to overcome the surface tension and elasticity of the 5% concentrated medium used in this study. Therefore, it is possible that when the drops of cells were placed onto the solid surface of the agar, they exhibited this swarming phenomenon and became aggregated in the area where cells were “pushed” into the agar by the needle. This could explain why the images taken under the confocal microscope revealed localized areas of fluorescent intensity along the needle at a particular depth. Another explanation is that the rod shaped *E. coli* cells have more surface area and thus more room for binding units on the outsides of the cells. While *S. aureus* cells are cocci and might have “rolled” off the needle, rod shaped cells have more potential to adhere to the needle’s length and consequently be pushed further down into the agar. This could also explain why there were still an innumerable amount of bacteria found at the needling sites with the highest dilution of *E. coli*. It is likely that the cells grew in numbers and swam through the medium by adhering to the length of the needle. *S. aureus*, on the other hand, is a non-motile bacteria, thus it would be reasonable to assume that the amount of cells found in the agar would decrease at each depth of the agar along the needle insertion if observed through a confocal microscope using fluorescence microscopy.

Understanding the distinct behaviors that these bacteria exhibit in a fairly tensile, elastic environment can provide insight into the possible risk of infections caused by *S. aureus* and *E. coli* when practicing dry needling. On average, 2-8 million Staphylococcus organisms are required to cause an infection at an insertion site (Elek and Conen 1957) and a 0.29mm needle pushes about 660 *S. aureus* bacteria through the skin (Hoffman 2001). This implies that with a standard dry needling insertion, the amount of *S. aureus*

cells that are pushed down into the deep tissue of the skin can be compared to the amount of cells transferred via the needle using an overnight culture dilution between 1:100 and 1:500 yielding an absorbance range between approximately 0.003-0.012 nm under a 600 nm optical density (**Table 4**). The 1:500 is almost three times more diluted than the 1:200 (**Figure 2**) tube, which only showed 28.09% of growth along the needle insertion site. Thus, this data demonstrates the extremely low risk of acquiring an *S. aureus* infection into deep tissue via a dry needle and helps to anticipate the sorts of challenges an elastic, solid surface might present for bacterial transmission through the skin. Although *E. coli* only accounts for 7% of complicated skin infections, its highly flagellated morphology and subsequent ability to move easily through solid mediums imply that they could cause a deeper infection into the skin tissue if there were a high concentration of *E. coli* cells at the insertion site. Another possible explanation for the prominence of *E. coli* growth along the stab could be that it is a transient microbe on the skin surface, meaning that it is more amenable to being “picked up” and transmitted to another location—including through skin into deep tissue.

Though the results of this study did not support my hypothesis that both bacteria would experience inhibited growth in highly concentrated media, they did provide useful insight about the behavior and movement of these bacteria in a relatively elastic environment and at each depth of a needling site. Additionally, the study served to further validate the hypothesis made in a few other studies that the risk of infection from dry needling is tremendously low, and sterilization of the skin prior to needle insertion is a precautionary measure that is not necessary for the prevention of infection in deep tissues.

## LIMITATIONS AND FUTURE RESEARCH

The purpose of this study was to determine the potential risk of infection via dry needling by studying the approximate amount of bacterial cells that could be transferred into an agar that mimics the elastic modulus of human skin. The use of agar generates a highly controllable environment that provides temperature control, good visualization and clarity of bacterial growth, and a sterile environment that lacks unwanted toxic bacterial inhibitors. While using agar allowed for a controlled environment the experiment was limited to using an agar concentration of 5.0%, which is nowhere near the elasticity of actual human skin. Furthermore, because *S. aureus* is part of the skin's micro flora and can reside on the skin surface for longer periods of time, the cells on the agar surface did not truly mimic the adherence of *S. aureus* to the surface of the skin. There were also complications regarding the preparation of the agar. Between manually pipetting the agar into the test tubes and heating them in the autoclave, bubbles began to form in the media, which rose to the top of the tube and left indentations on the agar surface. If a drop of cells was placed inside an indentation, this could have caused a decrease in the surface area covered by the drop of cells, which would have in turned created error in the calculation of the amount of cells that were actually transferred into the agar. This could explain the large amount of cells that are visualized in the confocal images. Moreover, the confocal microscopy proved to be more complex and difficult to navigate than anticipated. When using the Green Fluorescent Protein laser, the agar itself contained green, fluorescent properties, making it difficult to discern which part of the images were bacteria and which part was background fluorescence from the agar.

For future research, it would be ideal to obtain a raw chicken breast (or some other type of animal meat that could be purchased from a grocery store) that has a much similar strength and elasticity to human skin and muscle tissue, and perform the same experiment with an inoculated meat and skin core. This would not only be much more similar to human skin than agar, but would also completely eliminate the need to prepare a medium in the laboratory. Also, the use of fluorescent microscopy could be improved with the use of a more specific reporter gene for *E. coli* such as a direct fluorescent antibody for more precise visualization.

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## APPENDICES

### Appendix 1: Sample Calculations

Calculations used to determine amount of cells transferred via needle

Diameter of 10 mL screw thread tube	10 mm
Diameter of 5 $\mu$ L drop of cells	5 mm
Diameter of needle	0.3 mm

**Area of drop of cells**

$$\pi r^2 = \pi (2.5 \text{ mm})^2 = 19.63 \text{ mm}^2$$

**Area of needle**

$$\pi r^2 = \pi (0.15 \text{ mm})^2 = 0.07065 \text{ mm}^2$$

**Percentage of drop area covered by needle**

$$0.07065/19.63 \times 100 = 0.360\%$$

1)  $CFU's/mL = 79/10^{-8} = 7.9 \times 10^9$

2)  $CFU's/\mu L = 7.9 \times 10^9/1000 = 7.9 \times 10^6$

3)  $CFU's \text{ in } 5\mu L \text{ drop} = 7.9 \times 10^6 \times 5 = 3.95 \times 10^7$

4)  $CFU's \text{ transferred via needle} = 3.95 \times 10^7 \times 0.0036 = 1.42 \times 10^5$

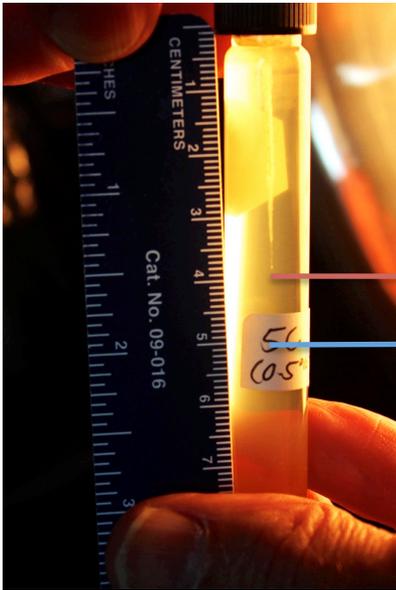
**Appendix 2: Needling Growth in Tubes**

Experiment 1 – September 2015

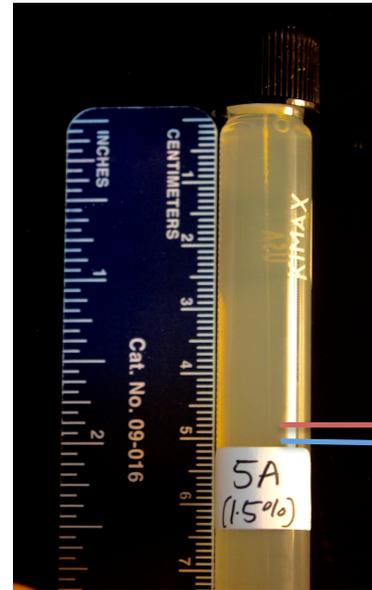
Varying agar concentrations at 1:2 dilutions of overnight *S. aureus*

— Total Depth of Stab  
— Total Depth of Significant Growth

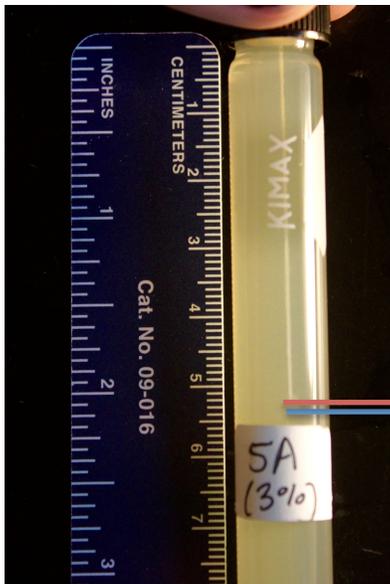
(only one replicate from each concentration was photographed)



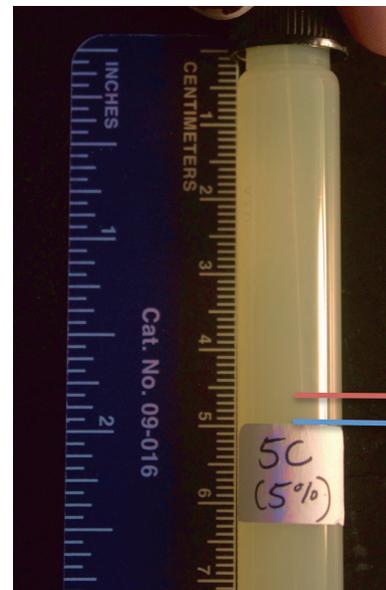
0.5% agar



1.5% agar



3.0% agar

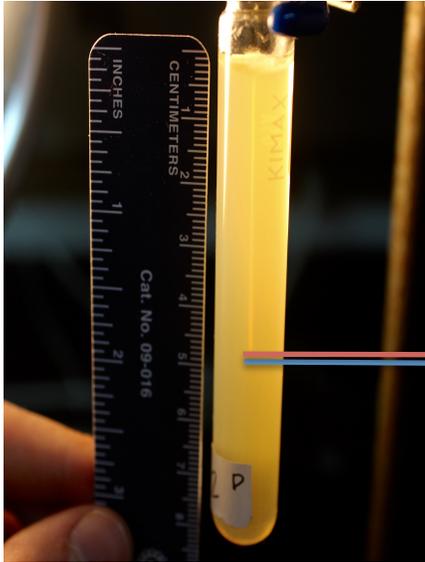


5.0% agar

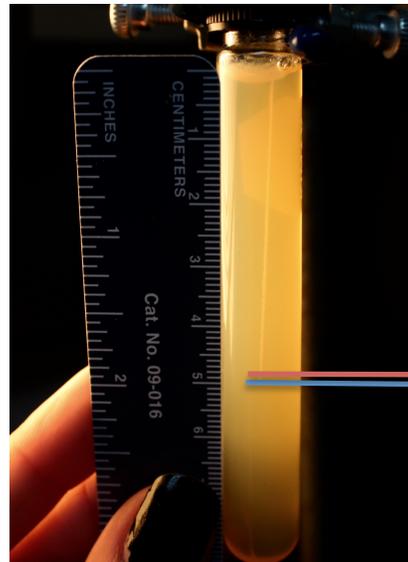
Experiment 2 – October 2015

Varying dilutions of overnight *S. aureus* with 5% agar concentration

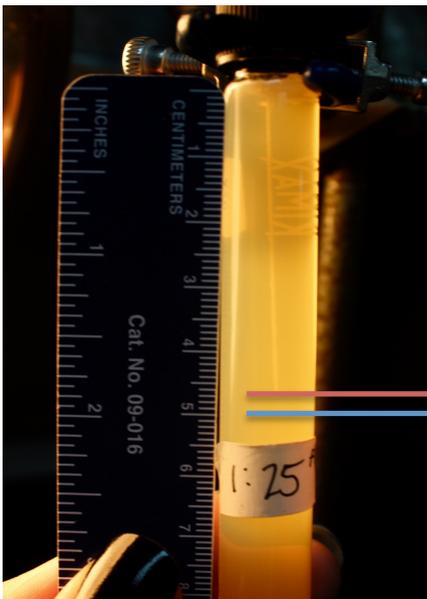
— Total Depth of Stab  
— Total Depth of Significant Growth



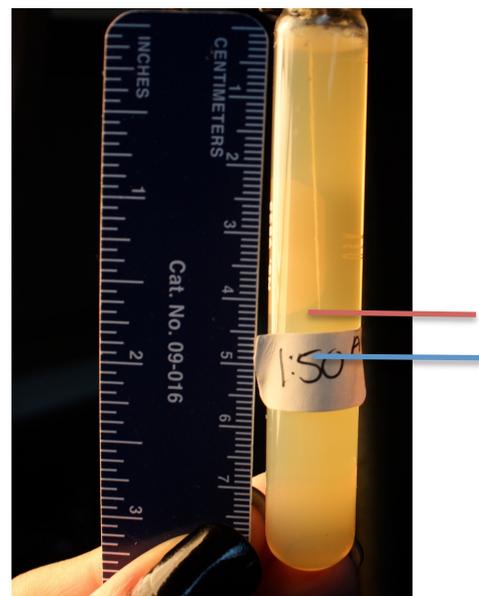
1:2 dilution



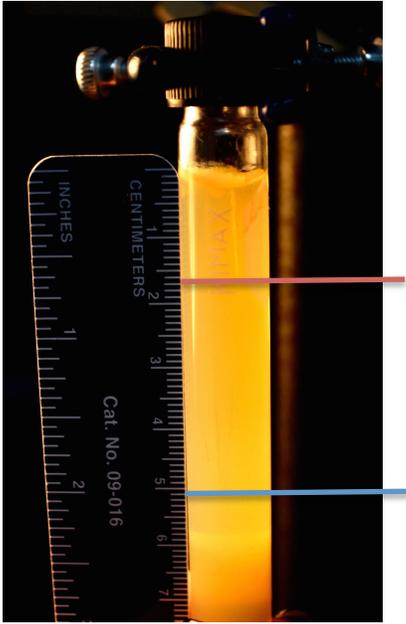
1:10 dilution



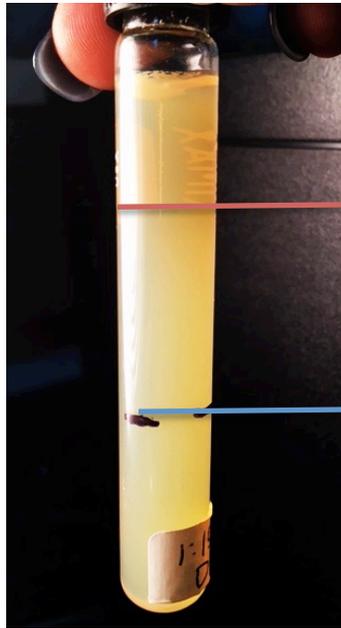
1:25 dilution



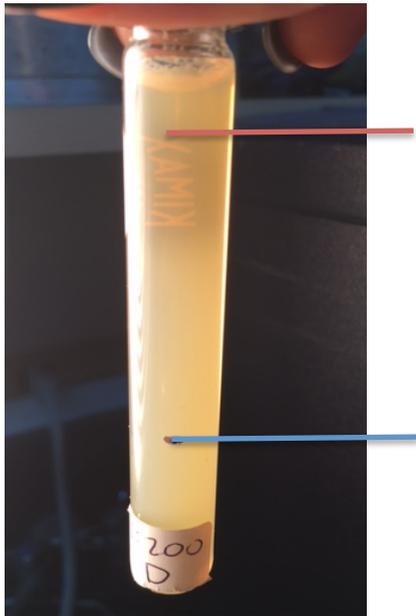
1:50 dilution



1:100 dilution



1:150 dilution



1:200 dilution

Experiment 3 – January 2016

Varying dilutions of overnight *E. coli* with 5% agar concentration

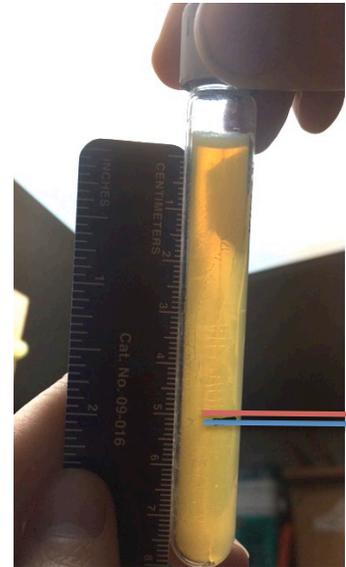
— Total Depth of Stab  
— Total Depth of Significant Growth



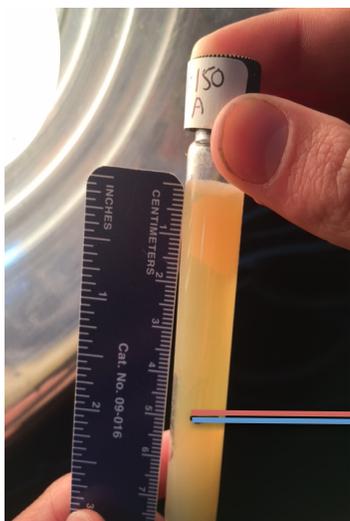
1:25 dilution



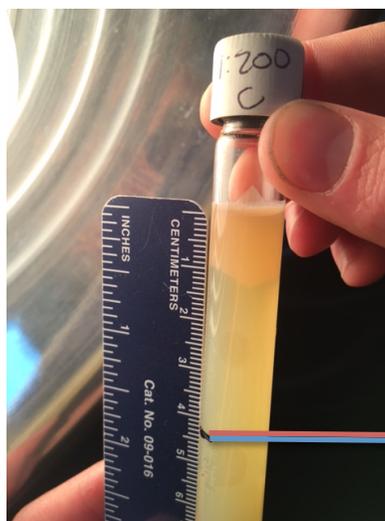
1:50 dilution



1:100 dilution

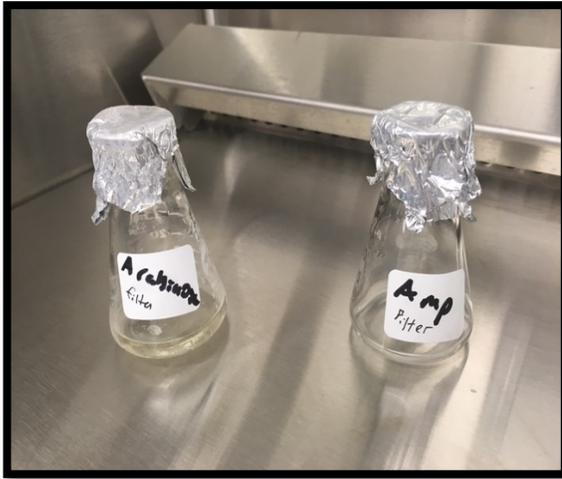


1:150 dilution



1:200 dilution

**Appendix 3: Preparation of Amp/Arab Media**



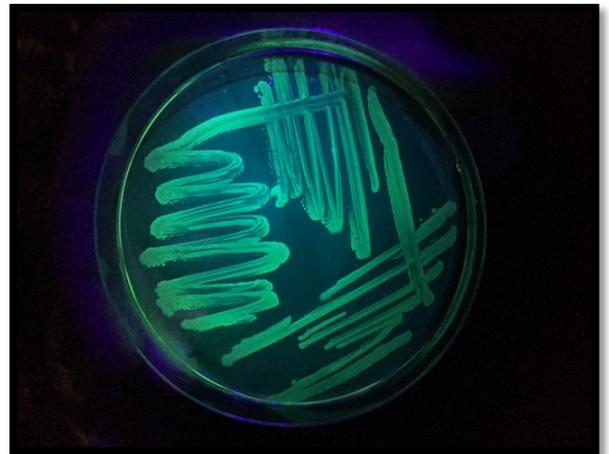
Filter sterilized Ampicillin and L-Arabinose



Manual pipetting of 5% agar



Amp/Arab TSA plates for *E. coli* fluorescence

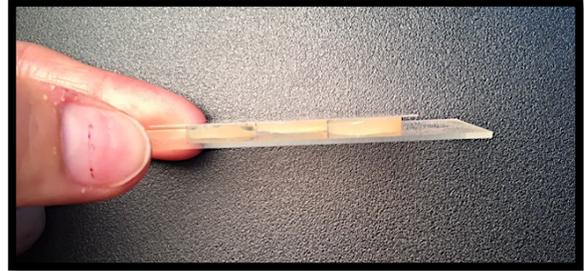


*E. coli* fluorescence under UV light

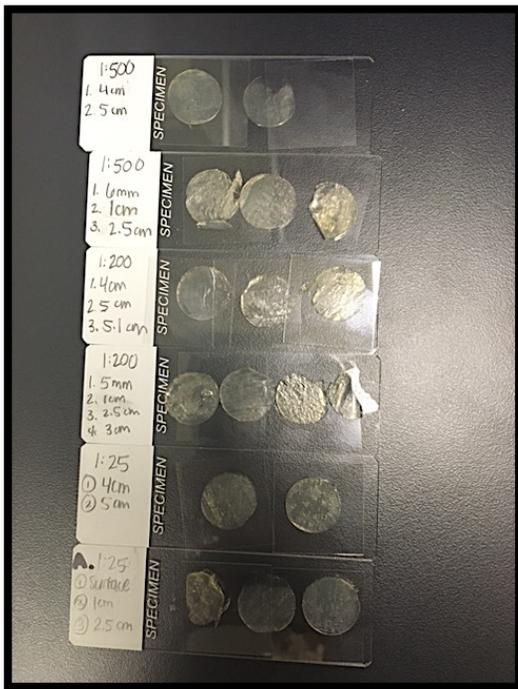
**Appendix 4: Preparation of Agar Slices for Confocal**



Top view of agar slices



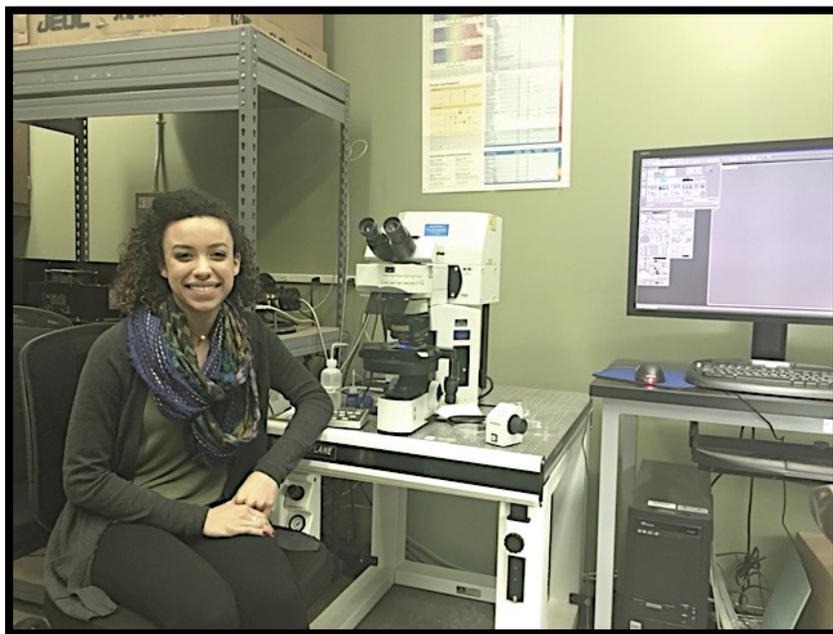
Side view of agar slices



Agar slices on slides



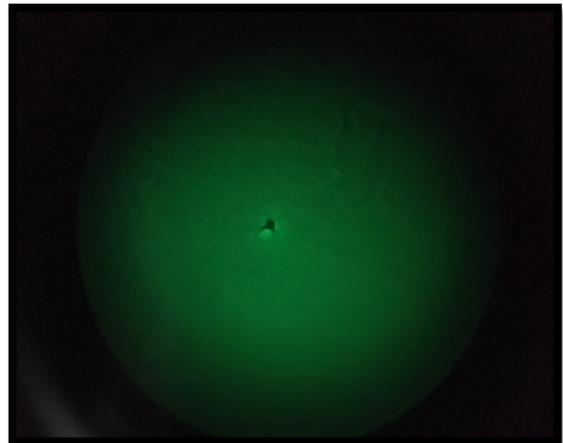
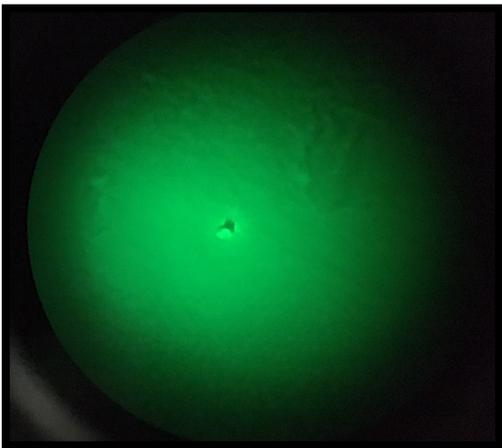
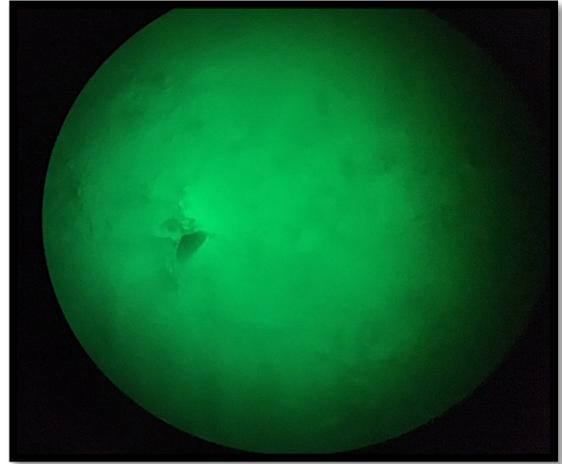
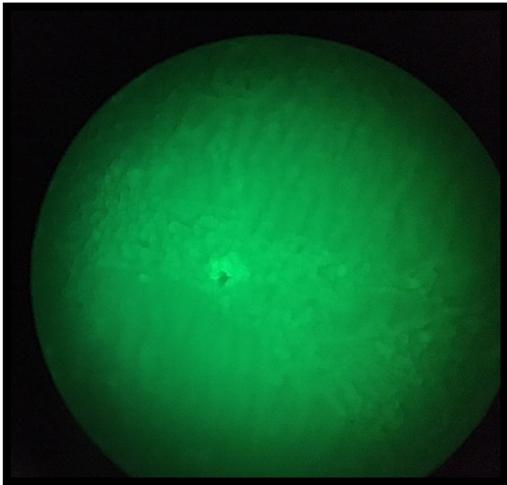
Stack of slides



Student Denisse Breton preparing to use the confocal microscope

**Appendix 5: Eye Lens Viewpoint**

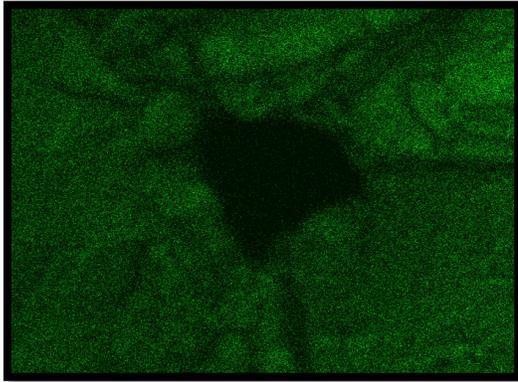
The images below show some needle insertion sites visualized with the ocular lens magnification of 10 X. Areas of brighter luminescence along needling site indicate presence of *E. coli* bacteria. These holes were localized, centered, and then viewed using 40 X magnification in **Appendix 6**.



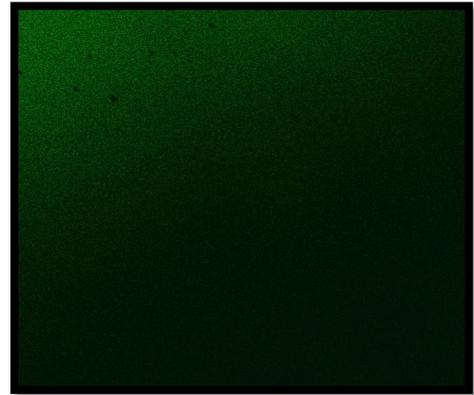
**Appendix 6:** *Confocal Images with E. coli*

Below are images taken of the needle insertion site at each dilution and depth of agar under a total magnification of 40 X.

1:25 Dilution



A. 1 cm

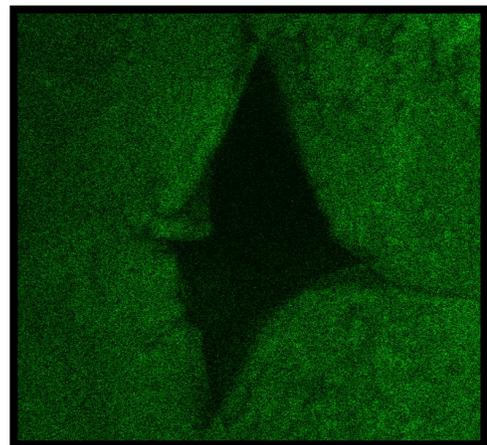


B. Comparison of cells present vs. absent

1:50 Dilution

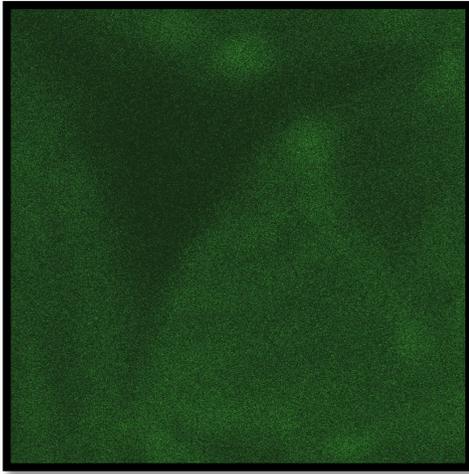


C. 2.5 cm

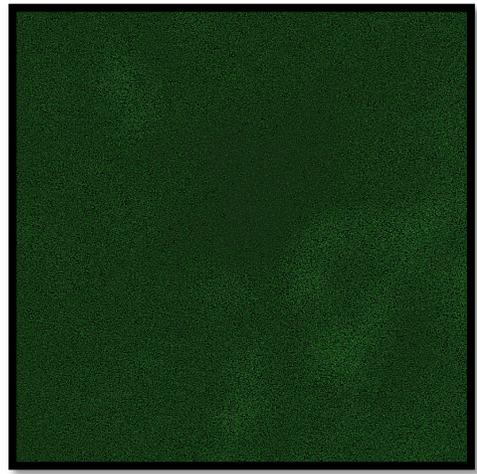


D. 4 cm

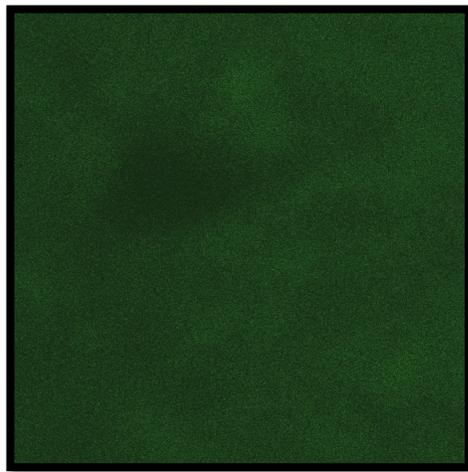
1:100 Dilution



E. 1 cm

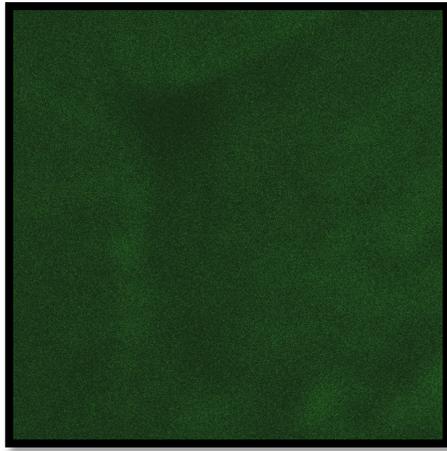


F. 2.5 cm

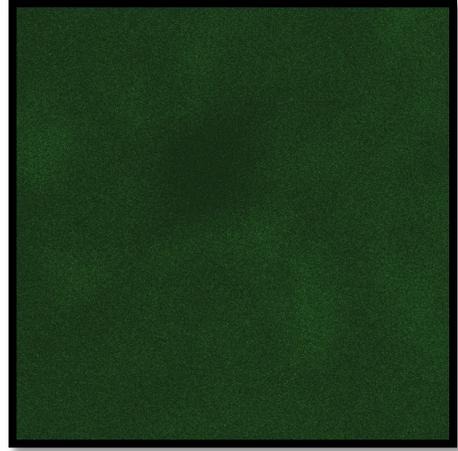


G. 5.0 cm

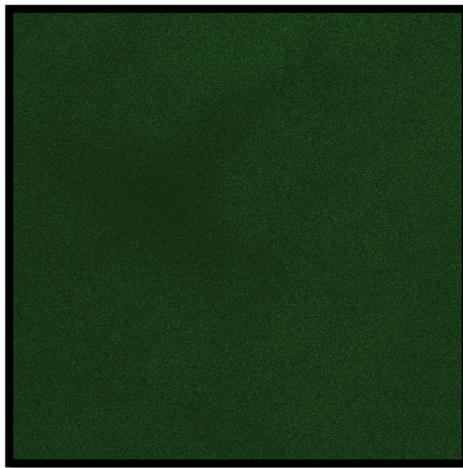
1:150 Dilution



H. 1 cm

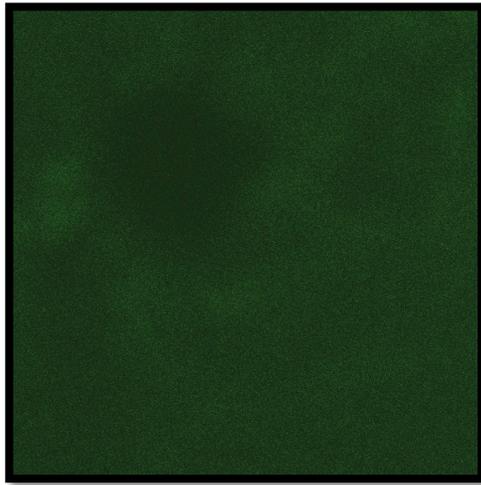


I. 2.5 cm



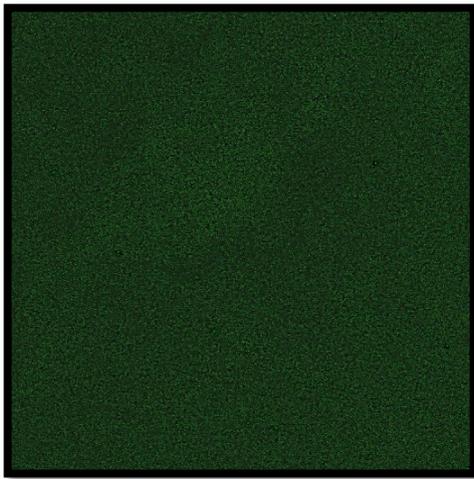
J. 4 cm

1:500 Dilution

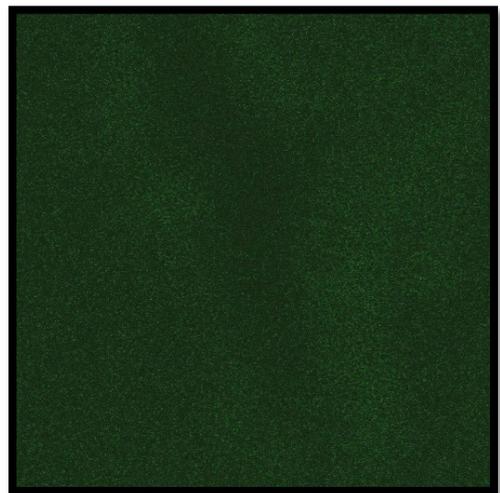


K. 4 cm

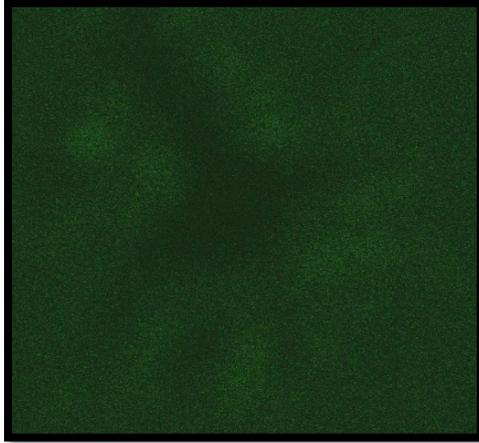
1:1000 Dilution



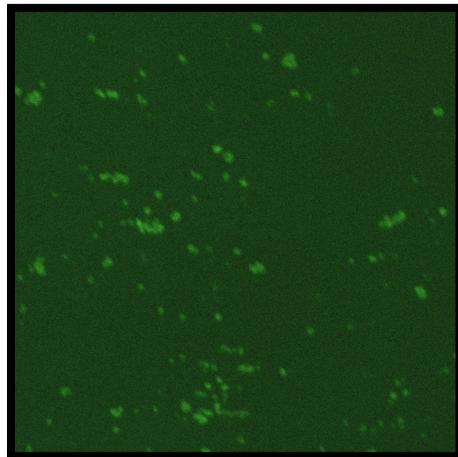
L. 1 cm



M. 2.5 cm



N. 4 cm



O. 5 cm – 600x objective – Individual *E. coli* cellse