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FACIAL MICROBIOTA AND ANTIBIOTIC RESISTANCE: A COMPARATIVE STUDY OBSERVING THE PRESENCE OF RESISTANCE GENES AND DIMINISHED SUSCEPTIBILITY

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DIMINISHED SUSCEPTIBILITY

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An Abstract of the Thesis by
Kayla Stricklan

The looming threat of increased antibiotic resistance leading to a post-antibiotic world is progressively becoming a reality. A significant portion of the fight against antibiotic resistance development is slowing down the prescribing of antibiotics for unnecessary conditions while simultaneously encouraging stricter adherence to antibiotic course completion. One of the often-unnecessary conditions is acne vulgaris. Though the disease may be unsightly and discouraging, it does little to affect overall health of the individual with the exception of psychosocial affects. Utilizing facial swabs from 144 participants, samples were tested for the colonization of *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis* as well as the presence of resistance genes *Cerm(X)* and *Tet(M)* using polymerase chain reaction and gel electrophoresis. Amplification of the samples portrayed that *Propionibacterium acnes* was the most prevalent microbe present, even from samples obtained from students with minimal blemishes. The second most common was *Staphyloccocus epidermidis* and few samples tested positive for *Staphylococcus aureus*. Amplification of resistance genes demonstrated fair amounts of resistance present, even in participants who had not taken antibiotics in the last two years. Out of the 144 initial samples, 60 isolated samples were utilized for susceptibility testing and the first set determined a slight decrease in

diameter of the resulting zone of inhibition when exposed to clindamycin, doxycycline and tetracycline, but not rifampin.

A subsequent 42 samples were collected for susceptibility testing using direct colony suspension. Results from testing demonstrated a significant decrease in the zones of inhibition between those who had not taken antibiotics and those who had taken two types of antibiotics for general illnesses and acne. The differences between tetracycline, clindamycin, rifampin and doxycycline were 4.64, 6.34, 11.86 and 7.66-millimeters, respectively with significant p-values between rifampin and doxycycline suggesting that the development of resistance is more prevalent in rifampin than previously determined.

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CHAPTER I

INTRODUCTION

Antibiotic Resistance: A Global Problem

Antibiotic use is on a continuous rise while the development of new antibiotics has reached a drastic halt. Combine both phenomenon with bacteria that are perpetually evolving and the world is facing a global crisis. Antibiotics have been likened to a four-edged sword against bacteria because they are capable of benefitting both the individual and the community by preventing the spread of illness but these benefits come at the cost of communal antibiotic resistance development and the collateral damage affecting one's microbiota (Yatsunenko et al. 2012). Although antibiotics have existed in nature for hundreds of millions of years, they have become a cornerstone of medicine in the decades since the Second World War (Davies, 2010). It is widely known that Alexander Fleming ignited the search for competing antibiotics with his discovery of penicillin in 1928. Since then, the magnitude at which antibiotics are both prescribed and utilized has exploded and may be attributed to the perception that antibiotics are entirely safe (Blaser, 2016). However, looking specifically at agriculture, evidence suggests that the use of small doses of antibiotics promotes growth in livestock and affects metabolic development (Zimmerman, 1986).

Further research has demonstrated an increase in a wide-array of comorbidities caused by antibiotic use including but not limited to obesity, diabetes, celiac disease and asthma. In mice, antibiotic exposure led to effects in adiposity and bone growth as well as altered normal immunologic development (Cho et al., 2012). Aside from these, the biggest side effect of antibiotic use is the potential for development of antibiotic resistance, which is often overlooked and demands more attention. Antibiotics are incredibly vital and should not be completely avoided. It is imperative that more accurate antibiotics be developed for targeting individual pathogens to minimize damage and preserve communal structure and function in the healthy microbiome rather than bombarding and forcing germs into submission with broad-spectrum antibiotics (Blaser, 2016).

The development of resistance is evolutionary and inevitable even when used properly (O'Neil, 2014). An integral problem with antibiotic use is the misuse and abuse when both consuming and prescribing them. Controlling the excesses may stabilize the situation but cannot reverse the deterioration that has likely occurred (Clemente et al., 2015). Globally, consumption of antibiotics in human medicine had increased by nearly 40 percent between 2000 and 2010. The most recent estimates suggest that deaths attributed to antimicrobial resistance may rise from the current estimate of 700,000 lives per year to ten million lives annually by 2050 (O'Neill, 2014). Because antibiotics are a mainstay in medicine, a world without their effectiveness would be dismal at best. Just about every ailment would be affected ranging from more severe medical

necessities like surgery and cancer treatment using chemotherapy to typical, less-severe and more widespread issues like acne vulgaris.

Propionibacterium acnes: History and Microbiological Implications

The "bacillus of acne" that is currently known as *Propionibacterium* has not always been classified under the genus. Unna discovered *P. acnes* in 1896 while researching blackheads. In 1923, the bacterium was mistakenly placed under the genus *Corynebacterium*. Although the two are closely related, they differ in catabolic processes and tolerance of oxygen. Ten years later, the bacterium was correctly classified (Douglas & Gunter, 1946). The genus *Propionibacterium* is named for the ability of some of its species to synthesize propionic acid, making them useful in the production of various compounds in the industrial manufacturing of probiotics and cheese (Falentin et al., 2010). Commonly a gram-positive anaerobic bacillus residing in the sebaceous follicles of the human skin, *P. acnes* has also been found in the oral cavity, intestinal tract, and the external auditory canal (Grice & Segre, 2011). When discovered in these locations, the bacteria are commensal, but the microorganism has been blamed for opportunistic infections in cerebrospinal fluid shunts, breast implants, cardiovascular devices and prosthetics (Piper et al., 2009).

Propionibacterium acnes is an aerotolerant organism, found to be able to tolerate oxygen for a few hours. If allowed to grow anaerobically, *P. acnes* will survive for up to eight months in vitro and can survive for extended periods of time in human tissues that have low oxidation potentials (Csukas, Banizs and

Rozgonyi, 2004). The microbes are slow-growing, usually requiring 7-14 days of incubation. They can also resist phagocytosis and have the ability to persist in macrophages (Webster et al., 1985). Its ability to survive for extended periods of time may be attributed to its capacity to produce biofilms, a thin and slimy film of bacteria that adheres to the surface of the organism, conferring protection. To characterize *P. acnes*, biochemical tests, as well as susceptibility tests, are recommended. The bacterium is catalase positive, nitrate reductase positive and indole positive in 70 percent of samples. It is resistant to metronidazole and fosfomycin (Aubin et al., 2014).

Acne and Antibiotic Resistance Development

While there are much more severe conditions that require antibiotic intervention, one of the most common diseases requiring treatment is acne vulgaris, a skin disease affecting up to 50 million individuals in the United States (Zeichner, 2013). The highest prevalence of the disease can be found in people between the ages of 12 and 24 with approximately 85% of the population affected (Harrop et al., 2007). Acne vulgaris will manifest in a variety of ways with the initial lesions presenting as open and closed comedones, commonly referred to as blackheads and whiteheads, respectively. The open comedo is exposed to the skin's surface and exposed to oxidation which converts the sebum to a grayish or black color (Fife, 2009). Closed comedones, on the other hand, are composed of a collection of sebum, keratinocytes, and microorganisms responsible for blocking the follicular opening. While comedones are considered

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non-inflammatory, other lesions typical of acne vulgaris are inflammatory. These lesions are commonly referred to as pustules, papules, nodules or cysts. Patients with a few comedones are considered to have mild acne while those with many comedones are deemed as moderate. These types of patients are typically able to treat their lesions with topical retinoids. Severe patients have acne that presents as both inflammatory and non-inflammatory lesions. It is in the moderate-severe range that patients should seek antibiotic medication. Further issues arise when patients use topical or systemic antibiotics alone as monotherapy significantly increases the development of antibiotic resistance (Fife, 2009). The addition of benzoyl peroxide in combination with oral or topical antibiotics significantly reduces antibiotic resistance development because it acts to wash out the colonizing, resistant bacteria (Gollnick et al., 2003).

The skin is the largest human organ and is composed of many types of commensal bacteria. Two of the most predominant species found colonizing the skins surface are *Propionibacterium acnes* and *Staphylococcus epidermidis*, followed by the presence of *Staphylococcus aureus*, *Micrococci*, *Corynebacterium spp*. and several other genera. *Propionibacterium* colonizes the lipid-rich pilosebaceous skin follicles and are thought to be the primary cause behind the development of the multifactorial disease acne vulgaris. The disease is multifactorial because all factors including increased sebum, ductal epidermal hyperproliferation, colonization of *P. acnes* and inflammation contribute to disease (Harper and Thiboutot, 2003). Although the species is considered commensal with potential for health-beneficial side effects, there is much

evidence to suggest that *P. acnes* acts as an opportunistic pathogen as well (Perry & Lambert, 2011). The production of chemotactic factors and proinflammatory interleukins like IL-2 are mainly responsible for the inflammatory phase of acne vulgaris. Interleukin-2 acts as a pro-inflammatory mediator, recruiting T lymphocytes, neutrophils and foreign body giant cells (Leyden, Del Rosso and Webster, 2007). *Propionibacterium acne* possesses the ability to metabolize sebaceous triglycerides into fatty acids, which also attracts neutrophils and activates complement (Webster, 2002).

Antibiotic Resistance Mechanisms in Microorganisms

Although the topic of antibiotic resistance is an ancient one as evidenced by the emergence of the serine beta-lactamases over two million years ago, it carries much more weight in today's society (Hall & Barlow, 2004). The development of resistance in microorganisms can occur in an innumerable amount of ways. A population of bacteria may become resistant once they accumulate several mutations or by the acquisition of specific point mutations. They may also acquire resistance genes that protect the cell from antibiotic destruction. This resistance can be developed by genetic elements like transposons and plasmids that have the potential to transfer from strain to strain within the resident skin flora (Swanson, 2003). Plasmids act as platforms for the exchange of genes, allowing for reassembly and assortment, which gives bacteria the ability to collect potentially useful genes that enable them to expand into regions that were previously too hazardous for them (Bennett, 2008).

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Transposons are systems of mostly random jumping genes that have the ability to incorporate resistance genes within their element either intramolecularly or intermolecularly, possibly moving from a plasmid to a bacterial chromosome and vice versa (Craig, 1997).

Bacterial resistance can be conferred actively in intricate manners, specific to the antibiotic target. A particular enzymatic strategy employed by bacteria in response to antibiotic pressure is hydrolysis. During hydrolysis, enzymes cleave vulnerable bonds crucial to their biological activity and in doing so; destroy their integrity (Wright, 2005). Phenotypic resistance can be caused by the enzymatic activation of an antibiotic, which then modifies the antibiotic target and prevents the accumulation of intracellular concentrations becoming large enough to kill the cell by way of efflux pumps (Allen, 2010).

Acne Specific Antibiotics and Resistance Development

Acne vulgaris often requires antibiotic intervention. Though, not a fatal disease demanding emergency attention, the appearance of acne has been associated with significant psychosocial burdens, which greatly affect the quality of life in a negative manner (Tan, 2004). In fact, several studies have equated the battle with acne to have an emotional impact similar to disabling diseases like diabetes and epilepsy (Knutsen-Larsen et al., 2012). Because of this, acne is the most common disorder treated in dermatology practice, accounting for 8-9 million oral antibiotic prescriptions alone in the United States. The majority of which are broad-spectrum tetracycline derivatives, specifically doxycycline because of its ease of absorption and long half-life (Del Rosso, 2007). Other antibiotics that

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have been used include macrolides like clindamycin and erythromycin, trimethoprim/sulfamethoxazole, benzoyl peroxide as a combination and topical antimicrobial as well as the recently emerging rifampin. These lipophilic antibiotics are the preferred drug of choice because although *P. acnes* demonstrates susceptibility to a wide array of antibiotics in vitro, in vivo they are the only drugs able to penetrate the lipid-filled microcomedones (Leyden, 2001).

Both oral tetracycline and erythromycin were available for treatment in the early 1950s, closely followed by doxycycline in 1967 and minocycline in 1972 (Leyden, 2004). Early research in favor of the development of antibiotic resistance actually proved to be null and void. The first evidence of antibiotic resistance in vitro and in vivo to both erythromycin and clindamycin was discovered shortly after the introduction of topical agents, in 1979 (Crawford et al, 1979). Though the study only included 22 patients, a demonstration of antibiotic resistance in twenty percent could not be ignored. Adding to this research in 1983, Leyden and his colleagues proved that prevalence of antibiotic resistance was commonplace among those receiving long-term treatments of both tetracycline and erythromycin (Leyden, 1983). Additionally, in the late 1980-early 1990s, an extensive study at Leeds discovered more clinically significant antibiotic resistance as well as strains of bacteria that were resistant to multiple drugs (Eady et al., 1993). A link between topical antibiotics and the development of antibiotic resistance has yet to prove that they are the integral cause of increased prevalence though several studies have nodded to the theory. A ten-year study from 1992-2001 in particular observed the prevalence of resistance to

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erythromycin and clindamycin in comparison to resistance caused by tetracycline. Resistance to erythromycin and clindamycin was more prevalent, demonstrating a possible link between topical agents and increased development of resistance (Coates et al., 2002).

A current estimate from data collected from five continents demonstrates a widespread and disturbing epidemic of *P. acnes* cases showing resistance to treatment with antibiotics (Dreno et al., 2014). A multitude of studies suggest the development of antibiotic resistance occurs in as many as 50 percent of cases following treatment with oral or topical antibiotics (Esperson, 1998). A positive relationship exists between antibiotic resistance development and lengthier treatment plans (Tan et al., 2001). The use of antibiotics for long-term periods is to blame for selective pressures whereby antibiotics eliminate the susceptible bacteria while the antibiotic-resistant bacteria is left behind to continue proliferation (Patel et al., 2010). While some treatment plans suggest that a 6 to 8-weeks course of antibiotics is perfect, other sources indicate that a patient must be treated for six months to avoid the establishment of antibiotic resistance. (Mills, Thornsberry et al. 2003). Further research details that *P. acnes* is not treated as a pathogen in the healthy individual and that resistant strains do not place them nor their contacts at severe risk for difficult to treat, systemic *P. acnes* infections. The problem lies within the commensals carried by humans that have the potential to become pathogenic as they can develop resistance to the antibiotics used in acne treatment (Patel, 2010). Antibiotic use in acne treatment

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can also negatively impact the normal, protective flora found on the skin predisposing the body to colonization by opportunistic pathogens.

Both doxycycline and tetracycline are first choice antibiotics in the battle against acne vulgaris though doxycycline is preferred because it has proven to be more effective with an earlier clinical response (Leydon, 2009). Tetracyclines act by binding reversibly to the 30S ribosomal subunit and interfering with the binding of the tRNA-amino acid complexes, ultimately inhibiting protein synthesis (Bailey et al., 2014). Mutations in the gene encoding 16S rRNA have also been implicated as another cause of resistance development (Eady, Gloor and Leyden, 2003). Tetracyclines are considered broad-spectrum because they are effective against gram positive and negative bacteria as well as several intracellular pathogens and some protozoa. Resistance is conferred when there is a decreased penetration of the antibiotic into the bacterial cell. Efflux systems activate and flush antibiotics out of the cell, preventing accumulation. Protection of the bacterial 30S ribosomal subunit against antibiotic binding is often accomplished by the *tet(M)* gene (Corso et al., 1998). *Tet(M)* resistance is often achieved by way of transposons (Doherty et al., 2000). Other mechanisms of resistance include altering the ribosomal target site and enzymatic modification of the antibiotic (Mayers, 2009).

Thought to be a secondary line of treatment behind tetracycline, macrolides are a family of antibiotics that inhibit protein synthesis by preventing polypeptide elongation. In order to be effective, macrolides bind reversibly to the 23S ribosomal RNA found on the 50S ribosomal subunit (Murray et al., 2016).

Resistance to erythromycin occurs once methylation of 23S rRNA takes place. This methylation prevents initial binding of the antibiotic, therefore hindering its efficacy. Alternative methods of resistance are conferred by enzymatic inactivation of the macrolide as well as point mutations encoding the V domain of the peptidyl transferase loop of 23S rRNA and ribosomal proteins (Ross et al., 1997). Often times, target site alteration is encoded by the *erm(X)* gene (Ross et al., 2003). Point mutations occurring at site *E. coli* equivalent nucleotide base 2058 in *P. acnes* confers cross-resistance to both erythromycin and clindamycin (Coates et al., 2002). Many studies have already emerged demonstrating the evidence of high levels of antibiotic resistance found in macrolides. This phenomenon can be attributed to the long-term treatments that are often required in acne therapy (Ross, 2001). Another disturbing phenomenon surrounding macrolide resistance is the cross-resistance between them and lincosamides as well as streptogramins due to similar binding sites in rRNA shared by the three classes of antibiotics (Fan et al., 2016).

Erythromycin resistance in *P. acnes* has been observed as early as 1989 when erythromycin-resistant *Propionibacteria* were isolated from skin surfaces of 51 percent of patients treated with oral erythromycin. Those treated with topical clindamycin demonstrated resistance at a rate of 42 percent while only three percent of those who were untreated displayed resistance (Eady et al., 1989). A double-blind study performed in 2003 enrolled 208 patients with acne vulgaris in a 24-week study to determine resistance rates associated with the use of topical two-percent erythromycin gel. Eighty-seven percent of *Staphylococci* samples

taken initially showed erythromycin resistance, which increased to 98 percent after 12 weeks, with no sign of regression twelve weeks post treatment (Mills et al., 2003). A study recently published in 2014 employing the use of multilocus sequence typing (MLST) deduced that antibiotic resistance to macrolide-lincosamide family of antibiotics was detected almost entirely throughout the sample of 76 patients. This resistance was attributed to the presence of point mutations found in 23S rRNA (Giannopoulos et al., 2014). An even more recent study performed in China, published in 2016, found combined resistance rates between macrolides and lincomycin to be extremely high at 47.8 percent (Fan et al., 2016). A study performed in vitro, examined samples from healthy skin and nodulocystic and pustular lesions taken from 100 patients, ranging from 18 to 24 years of age. This study revealed that *Propionibacterium acnes* was at least 50% resistant to both clindamycin and erythromycin. The study also demonstrated that rifampin was more inhibitory than any of the other antibiotics, including the combined effects of clindamycin and erythromycin with benzoyl peroxide (Hassanzadeh, Bahmani & Mehrabini, 2008).

Perhaps a newer emerging route of antibiotic treatment for acne is rifampin; a drug that prevents transcription by binding to DNA-dependent RNA polymerase and ultimately inhibiting nucleic acid synthesis (Murray et al., 2016). This antibiotic is considered a "gold standard" in the treatment against *P. acnes* because of its effectiveness against biofilm formations (Furustrand, Trampuz, and Corvec, 2013). Combining rifampin with another antibiotic has previously been thought to delay the development of antibiotic resistance. Unfortunately,

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rifampin resistance is on a continual rise. In gram-positive bacteria, resistance is caused by a mutation in the chromosomal gene that codes for the beta subunit of RNA polymerase. This mutation, which takes place in the *rpoB* gene, reduces the ability of the antibiotic to bind to the enzyme (Furustrand, Corvec, and Betrisey, 2012). Gram-negative bacteria are intrinsically resistant due to decreased uptake (Murray et al., 2016). Rifampin has demonstrated an ability to decimate the production of biofilm in *P. acnes*, both in vitro and in vivo (Bayston et al., 2007; Furustrand et al., 2012). A study performed in 2013 described the substitutions that conferred rifampin resistance in *P. acnes*. Progressive exposure to rifampin produced a double-mutation, and ultimately, rifampin resistance emerged rapidly without combination therapy (Tafin et al., 2013).

Staphylococcus aureus **and** *Staphylococcus epidermidis*

The skin is not only colonized by *P. acnes* which begs the question, are other commensal microorganisms playing a role in pathogenesis? It has already been established that both *P. acnes* and *S. epidermidis* coexist along with other microflora within acne manifestations (Nishijima et al., 2000). But, recent estimates suggest that one-third to one-half of the general population with acne vulgaris are likely also to be colonized by *Staphylococcus aureus*, a gram-positive coccus residing on the skin (Fanneli et al., 2011). Described as one of the most common nosocomial pathogens, the bacterium is also ubiquitous and omnipresent. *Staphylococcus aureus* colonizes forty-fifty percent of the population as a whole, and the bacteria has a high ability to adapt and become

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resistant, leading to the increasingly common methicillin-resistant *Staphylococcus aureus* (MRSA) infection, which can already be found in approximately five percent of the community (Deleo et al., 2010). In fact, the development of MRSA in patients is a serious problem. In 2007, the Centers for Disease Control and Prevention (CDC) estimated an overall incidence of approximately 65,000 cases of invasive MRSA with about 15,000 classified as community-acquired (Klevins et al., 2007). These staggering statistics leave much concern regarding the development of resistance in both *S. aureus* and *P. acnes* (Delost et al., 2015). Though there is significant in vitro evidence implying that *Staphylococcus aureus* is a pathogenic contributor to acne vulgaris, there is also contradicting evidence to support *Staphylococcus epidermidis* (Hassanzadeh, Bahmani, and Mehrabani, 2008). In this study, significantly higher percentages of *S. aureus* were observed in the normal skin of both girls and boys compared to *P. acnes*. Aerobically, *Staphylococcus epidermidis* was isolated from 53 percent of the 100 samples, followed by *Micrococcus* at 45 percent and *S. aureus* at 41 percent. Anaerobically, *S. aureus* was isolated the most from 39 percent of the samples, followed by *P. acnes* at 33 percent and *S. epidermidis* from 21 percent (Hassenzadah, Bahmani, and Mehrabani, 2008). Further research suggests that prolonged use of antibiotics actually decimates the resident colonization of *S. aureus* (Delost et al., 2015).

Perhaps the most predominant pair colonizing the skin is *P. acne* and *S. epidermidis*. Because the two live in incredibly close proximity, their interaction has the potential to disrupt the delicate balance of the skin's ecosystem

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(Christensen et al., 2016). The possible disruption can contribute to the development of acne vulgaris (van Rensburg et al., 2015). While *P. acnes* exhibits a limited amount of strain variations but are still considered multiphyletic, *S. epidermidis* strains are even more heterogeneous than that. A study performed in 2016 compared the antagonism portrayed between the various strains found in both genuses on persons with acne-infected skin and those with healthier skin. The study found that two phylogroups of *P. acnes* increased antimicrobial activity against *S. epidermidis* while eleven out of 20 strains of *S. epidermidis* demonstrated the similar effects toward *P. acnes* (Christensen et al., 2016). Both observations are examples of interspecies competition suggesting that *S. epidermidis* may be utilized in the war on acne because of its evident anti-*P.acnes* activity.

Proposed Thesis Project

The purpose of this research was to initially isolate and identify the most common genuses of bacteria colonizing the face using standard polymerase chain reaction and primers specified for each of the following bacteria: *Propionibacterium acnes*, *Staphylococcus aureus*, and *Staphylococcus epidermidis.* Results suggest whether the prevalence of *Staphylococcus aureus* or *Staphylococcus epidermidis* or a mixture of the two in combination with *P. acnes* play a role in pathogenesis. Then, resistance genes commonly found among those bacteria who become resistant after interacting with antibiotics that are often used to treat acne vulgaris will also be used in polymerase chain

reactions to determine the amount of resistance in those who have and have not taken antibiotics. Selected resistance genes were C*erm*(X) and *tet*(M). In addition, this research utilized commonly prescribed antibiotics like doxycycline, clindamycin and tetracycline and compares their efficacy against the effectiveness of rifampin, utilizing Mueller-Hinton agar and the Kirby-Bauer method. Susceptibility to each antibiotic was recorded to determine the amount of resistance present in specific samples from each population, to determine if increased antibiotic use leads to a decrease in the diameter of the resulting zone of inhibition. Samples were also gram-stained to confirm morphology of the colonies isolated from participants in order to more accurately determine susceptibility. By separating participants in to three separate categories (**Table 1**), we may be able to observe whether antibiotic resistance is a direct cause of consumption or if resistance genes have been shared communally between the varying groups including those who have taken antibiotics specifically for acne in comparison to those who have taken antibiotics within the last two years for other illnesses vs. those who have not taken any antibiotics in the past two years.

Number of Students	Category
46	0-Students who have taken no antibiotics in the last two years
97	1-Students who have taken antibiotics for general reasons in last 2 years
8	2-Students who have taken antibiotics only for acne
35	3-Students who have taken antibiotics for both acne and general reasons

Table 1. Division of Participants. Participants were divided into groups based on use of antibiotics.

CHAPTER II

MATERIALS AND METHODS

Sample Collection

Samples were collected from 186 students attending Pittsburg State University in accordance with the standards and approval guidelines set forth by the Institutional Review Board (IRB). Prior to sampling, participants filled out a questionnaire detailing their history of antibiotic use for general illnesses as well as antibiotics taken specifically for acne, if applicable. Once applications were completed, 144 of the students' samples were collected using sterilized cotton swabs and distilled water. Three cotton swabs were used for each participant. Each of the cotton swabs were dipped into distilled water and used to swab problem areas of the face on each participant. A majority of the swabs were taken from the forehead along the hairline, the crevices surrounding the nares, and the chin. Once the cotton swabs were inoculated with the bacterial sample, two were placed directly onto reinforced clostridial media (RCM). The third was placed into 200 microliters of phosphate buffered saline or PBS buffer and placed in the freezer at -10 degrees Celsius. RCM plates were then placed in the incubator for 5-7 days under anaerobic conditions, using an Anaeropack System, 2.5-liter rectangular jar paired with a compatible satchet anaerobic gas

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generating system. After seven days of growth, samples were removed from the anaerobic jar and semi-pure colonies were utilized for isolation streaking and allowed to grow for an additional 5-7 days, dependent on growth. Once isolated colonies were available, a sterile inoculating needle was used to stab the colonies into diluted tryptic soy agar (TSA) preservation vials composed of one gram of tryptic soy broth (TSB) and 0.3 grams of granulated agar dissolved into 100 milliliters of distilled water. The samples were then refrigerated for preservation. The remainder of the isolated colony was utilized for gram staining.

Gram Staining Bacterial Samples

Each sample contained two-three variable colonies that were isolated and gram stained for identification. The glass slides were washed using Lava soap and dried with Kimberly-Clark Kimwipes EX-L. Upon drying, a dropper was used to place a single drop of distilled water on each slide. A sterile isolation needle was used to stab individual colonies that were evenly dispersed within the water drop and allowed to air-dry prior to being heat fixed to the slide. Once heat-fixed, the slides were flooded with Gram's crystal violet and allowed to stand for one minute and rinsed completely with distilled water. The slide was then flooded with iodine for one minute and rinsed with three to four passes of 95 percent ethanol, then rinsed with distilled water. Finally, the slide was flooded with safranin to counter-stain the bacterial cell; after sitting for a minute was once again, rinsed with distilled water. A Fisherbrand Bibulous Paper pamphlet was used to dry the slides prior to viewing under the microscope. Samples were observed under oil-

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immersion and classified as either gram positive or gram negative and for the presence of bacilli or cocci. A percentage of the four categories was gathered and gram negative bacilli samples were removed from experimentation. Several of the samples were further characterized to determine the genus and species of those bacteria that were recurrent.

Media Preparation

Several different types of media were utilized for this experiment. Blood agar was mixed for samples that did not grow well on Mueller Hinton agar, particularly those that required an anaerobic environment. To make the blood agar, 49.4 grams of Heart Infusion agar was dispensed into 950 milliliters of water and boiled to dissolve, prior to being autoclaved for 15 minutes. After cooling in a water bath set to 57 degrees Celsius, 50 milliliters of sheep blood was added to the solution and mixed on a stir plate, utilizing a stir bar. Plates were poured immediately, thereafter. Mueller Hinton plates were prepared for disc susceptibility because the starch included in the mixture protects against toxic material and controls the levels of inhibitors, especially those against tetracycline and sulfamides, which reduces interference with testing and allows for proper growth. The Mueller-Hinton mixture included two grams of beef extract, 17.5 grams of casamino acids, 1.5 grams of starch and 15 grams of agar, suspended in 1000 milliliters of distilled water. A liter of Mueller-Hinton plates, supplemented with 50 milliliters of sheep blood was prepared for samples that did not grow well on Mueller-Hinton alone. These plates were boiled for

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mixture, placed in the autoclave for 20 minutes, cooled in the water bath under the same conditions previously detailed and poured into plates at equal depths of four millimeters. Difco Reinforced Clostridial Medium was utilized for initial sampling because of its success in allowing the growth of *P. acnes* in previous research. Thirty-eight grams of the powder was mixed with 15 grams of agar and boiled, prior to being autoclaved and following the before-mentioned procedure.

Actinomyces broth was selected for liquid suspensions because it is ideal for the growth of *Actinobacteria*, which is the class under which, *Propionibacterium* are filed. In order to make Actinomyces broth, the following formula was followed: two grams of heart muscle infusion, 17 grams of pancreatic digest of Casein, 10 grams of yeast extract, five grams of sodium chloride, 13 grams of dipotassium phosphate, two grams of monopotassium phosphate, five grams of dextrose, one gram of ammonium sulfate, L-cystein hydrochloric acid and soluble starch, 0.2 grams of magnesium sulfate and 0.01 grams of calcium chloride. All ingredients were dissolved into one liter of distilled water and brought to a boil, prior to being poured into a one-liter flask. A Filamatic vial filler was used to evenly dispense five milliliters of sample in to each test tube, prior to autoclaving for 20 minutes.

Microbial Species Identification and Kirby Bauer Susceptibility

The refrigerated samples were set-aside for the duration of molecular amplification. Upon utilization of the tubes, the isolated colonies were transferred from the TSA tubes to Actinomyces broth using a sterile inoculating needle. Once

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suspended in culture, samples were allowed to grow for 2-3 days before they were examined. Upon examination, samples without growth were placed back in to the incubator for 2-3 more days, depending on culture. Samples with growth were labeled as A and B, if two different colonies were isolated. Each sample was streaked onto RCM for isolation using a sterile inoculating loop and placed back in to the incubator at 37 degrees Celsius to grow for two days. After two days, samples with visible growth were removed and an individual colony from each plate was removed for gram staining on a glass slide. Those without visible growth were re-swabbed onto RCM and placed in an anaerobic jar.

Actinomyces broth overnight suspensions of each sample were also used for Mueller-Hinton inoculation. A sterile cotton swab was dipped into the suspension and rolled onto Mueller-Hinton agar, completely covering the plate. Once inoculated, each plate sat for five minutes to dry prior to adding the BBL Sensi-Disc Antimicrobial Susceptibility test discs. Each sample was streaked onto two plates and divided evenly down the middle. On each side, an antibiotic disk was placed using sterilized forceps that had been dipped into alcohol and passed through the Bunsen burner prior to placing each antibiotic disc. The following disks were used for all samples: rifampin, clindamycin, doxycycline and tetracycline. Once the disks were placed, the plates were allowed to dry for five minutes prior to inversion and placement in the incubator at 37 degrees Celsius for 48 hours. Once 48 hours had passed, the zones of inhibition surrounding each disc were measured in millimeters and recorded.

Molecular Species Identification

      The samples placed in PBS buffer were utilized as the template for polymerase chain reaction amplification applying routine thermocycler conditions. The initial denaturation of the DNA sample was at 95 degrees Celsius for five minutes prior to 30 cycles through the following conditions: 95 degrees Celsius for 30 seconds, an annealing temperature varying between 45-68 degrees Celsius for 30 seconds and 72 degrees Celsius for 30 seconds. The final extension cycle was completed at 72 degrees Celsius for ten minutes, immediately followed by an infinite hold temperature at four degrees Celsius. A ten-picamole/lambda primer mixture was created from the working stock of each forward and reverse primer set. Twenty microliter solutions were prepared prior to being placed in the MyCycler thermal cycler. The concentration consisted of ten microliters of 2X Eco-Taq MasterMix, five microliters of the template and 2.5 microliters of both the forward and reverse primers. Four different sets of primers, ordered from Integrated DNA Technologies, were selected based on previous research summarized in Table 2 and were amplified using the same PCR parameters previously described with the only variation being the annealing temperature based five degrees below the boiling point of each set of primers. The first primer utilized was *Propionibacterium acnes* forward (5'-GCG TGA GTG ACG GTA ATG GGT A-3') and *Propionibacterium acnes* reverse (5'-TTC CGA CGC GAT CAA CCA-3') (De Morais Cavalcanti et al, 2011). The annealing temperature selected for *P. acnes* was 52 degrees Celsius. The primer selected for *Staphylococcus aureus* was SA2-F (5'-AAT CTT TGT CGG TAC ACG ATA

TTC TTC ACG -3') and SA2-R (5'- CGT AAT GAG ATT TCA GTA GAT AAT

ACA ACA-3') with an annealing temperature of 48.5 degrees Celsius (Saruta et

al, 1997). The primers utilized for *Staphylococcus epidermidis* amplification were

SE1-F (5'-ATC AAA AAG TTG GCG AAC CTT TTC A-3') and SE1-R (5'-CAA

AAG AGC GTG GAG AAA AGT ATC A-3') with an annealing temperature of 51

degrees Celsius (Pereira et al., 2010).

Primers	Primer Sequence
PacnesF	5'-GCG TGA GTG ACG GTA ATG GGT A -3'
PacnesR	5'-TTC CGA CGC GAT CAA CCA -3'
SA2-F	5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG -3'
SA2-R	5'- CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3'
SE1-F	5'-ATC AAA AAG TTG GCG AAC CTT TTC A-3'
SE1-R	5'-CAA AAG AGC GTG GAG AAA AGT ATC A-3'

Table 2. List of primers used for species identification

Molecular Resistance Gene Amplification

Primers selected for resistance gene amplification were placed in the PCR

Thermocycler under the same parameters used for species identification with the

exception of specific annealing temperatures. To test for the presence of the

erm(X) gene, the primer pair of Cerm 1 (5'-GAC ACG GCC GTC ACG AGC AT-

3') and Cerm 2 (5'-GGC GGC GAG CGA CTT CC-3') was used at an annealing

temperature of 57.5 degrees Celsius (Perez et al., 2010). This gene tested for

generic resistance to macrolides, lincosamides and streptogramins. The second

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resistance gene utilized was tet(M), composed of TetM-F (5'-ACA GAA AGC TTA TTA TAT AAC-3') and TetM-R (5'-TGG CGT GTC TAT GAT GTT CAC-3') (Seyfried et al., 2010).

Gel Electrophoresis

A two-percent agarose gel was utilized for gel electrophoresis. One gram of agarose was dissolved into 50 milliliters of 1X TAE buffer. The solution was boiled for 30-40 seconds until completely dissolved. Three microliters of ethidium bromide was added to the agarose, and was poured into a gel-casting tray for solidification. Two 15 well combs were placed at equal distance in the gel-casting tray. After 30 minutes, the gel-casting tray was placed in the gel electrophoresis apparatus and the agarose gel was completely submerged in TAE buffer prior to removing the combs. Eight microliter aliquots of each sample was pipetted into separate wells from left to right. Five microliters of 100bp DNA ladder with a 500ng/6microliter concentration was pipetted into the first lane in each comb. The positive and negative control for each bacterial identification reaction was pipetted into the second and third lanes of each comb, respectively. The sample was run at 100 volts for 30 minutes. Upon completion, the gel was removed and viewed under ultra-violet light prior to utilizing a FluorChem E to capture the images.

Subsequent Sampling

In order to confirm the results of the initial zones of inhibition recorded, a second round of samples was obtained. Two sterile cotton swabs were utilized and placed into distilled water. The first was used to swab in the same manner as before and placed directly onto RCM agar. The second swab was placed directly into Actinomyces broth. The RCM agar plates were allowed to grow aerobically at 37 degrees Celsius for 2-3 days depending on growth. This plate was utilized to obtain an initial sampling of colonies. The Actinomyces broth was allowed to grow in the same fashion, providing an anaerobic environment for *P. acnes.* Once the broth was fairly turbid, a sterilized inoculating loop was placed into the mixture and an isolation streak onto RCM agar was performed. A cotton swab was placed into the mixed Actinomyces culture and Mueller Hinton agar was utilized to perform disk-susceptibility on a mixed culture to observe the effect of the communal microbiota against antibiotics. Once colonies were isolated, half of colony was utilized for gram staining while the other half was used to create a direct broth suspension for antimicrobial Sensi-Disc susceptibility.

Mueller Hinton Antimicrobial Susceptibility Replication

The initial protocol for susceptibility testing was revised for the replication procedure in order to mimic the method suggested by BD BBL Sensi-Disc. Because a majority of the samples observed were gram positive, more specifically *Staphylococcus epidermidis*, a direct broth suspension was created using 3-5 similar colonies transferred into normal saline from RCM agar plates

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that had been incubated overnight. The (0.5) McFarland turbidity standard was strictly followed as dilutions were made if cultures became too turbid. Within 15 minutes of suspension, a sterile cotton swab was dipped directly into the sample and was rotated several times against the upper inside wall of the tube to remove excess fluid. The entire surface was streaked three times, rotating the plate 60 degrees between each inoculation. The lid was placed on top of the plate without completely covering to allow for 3-5 minutes of drying prior to the addition of the drug-impregnated discs. The discs were placed using forceps that were sterilized by ethanol and flaming, allowing for the Bunsen burner to burn off remnant between each disc. Doxycycline, clindamycin, rifampin and tetracycline discs were placed on to one plate at equal distances apart. The discs were allowed to sit for no more than 15 minutes prior to inversion and incubation at 37 degrees Celsius overnight. The resulting zones of inhibition were measured and recorded after 24 hours, instead of the initial 48-hour incubation.

Statistical Analysis

Statistical analysis was performed on each data sample. Mean values and standard deviations were calculated using Microsoft Excel. Box plots were analyzed using Alcula on-line box plot calculator. Quantile-quantile plots were performed to determined normalcy of data. Upon confirmation of normal distribution, a one-way analysis of variance (ANOVA) values were determined using Vassar Stats and double-checked against SPSS software. P-values were

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determined for each comparison and recorded, as well as post-hoc Tukey HSD

values to compare specific p-values between treatments.

CHAPTER III

RESULTS

Initial Sampling and Amplification

The initial 144 samples that were incubated anaerobically were utilized in five microliter aliquots and amplified to determine the presence or absence of *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis.* The comprehensive results can be visualized in Table 3 while the individual sampling results can be visualized in Appendix C. The size of the amplicon resulting from *Propionibacterium acnes* was 131bp and 132 out of the 144 samples were positive for presence of the bacteria. The amplicon from *Staphylococcus aureus* was 108 bp and only 15 out of 144 samples tested positive while 110 of the 144 samples tested positive for *Staphylococcus epidermidis,* with an amplicon of 124 bp.

Percentage of samples containing each experimental bacteria					
Propionibacterium acnes		*Staphylococcus aureus*		*Staphylococcus epidermidis*	
132/144	91.6%	15/144	10.4%	110/144	76.4%

Table 3 Comprehensive survey of facial microbiota.

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Resistance Gene Amplification

The same 144 samples were utilized in five microliter aliquots and amplified to determine the presence or absence of two common antibiotic resistance genes found in bacteria. The results are tabulated in Table 4. Samples were grouped based on consumption of antibiotics. The *tet(M)* resistance gene amplicon was 200 bp while the *Cerm(X)* resistance gene amplicon was 390bp. Thirty-six participants were placed in group zero, suggesting no antibiotic consumption in the last two years. In this group, 22 tested positive for *tet(M)* while 18 tested positive for *Cerm(X).* Participants placed in group one had taken antibiotics within the last two years for various illnesses. Out of the 74 participants in this group, 51 tested positive for *tet(M)* while only 31 tested positive for *Cerm(X)*. A very small subset of participants was placed into group two for consumption of antibiotics specifically for acne within the last two years. Five of them tested positive for *tet(M)* while three of them tested positive for *Cerm(X).* The last group consisted of 28 participants who had taken antibiotics for both acne and general illnesses within the last two years. In this group, 19 tested positive for *tet(M)* while 10 tested positive for *Cerm(X).* In totality, 61% of participants not taking any antibiotics tested positive for *tet(M)* and 50% tested positive for *Cerm(X).* The percentage of *tet(M)* presence increased by eight percent in participants taking antibiotics while presence of *Cerm(X)* resistance genes decreased nine percent.

Percentage of resistance genes present within each group of participants							
Group 0		Group 1		Group 2		Group 3	
Tet(M)	*Cerm(X)*	*Tet(M)*	*Cerm(X)*	*Tet(M)*	*Cerm(X)*	*Tet(M)*	*Cerm(X)*
22/36	18/36	51/74	31/74	5/6	3/6	19/28	10/28
61%	50%	69%	42%	83%	50%	68%	36%

Table 4. Antibiotic Resistance Genes. Comparison of the percentages of antibiotic resistance genes found within each group.

Susceptibility Testing

Once all of the resistance genes were amplified, isolated samples that were placed in TSA tubes were utilized to determine susceptibility. Samples from each group were randomly selected and the resulting zones of inhibition were compared against the guidelines outlined in the BD BBL Sensi-Disc Antimicrobial Susceptibility pamphlet, summarized in Appendix T. Out of the 60 samples in the first susceptibility testing, 22 were classified as resistant, while 8 were intermediately susceptible and 30 were susceptible to clindamycin. When comparing those results to the presence of *Cerm(X)* resistance genes, molecularly, 34 of the results were double confirmed while 26 were contradictory. These results can be summarized in Table 5.

Sample	Zone of Inhibition (mm)	Resistant ≤14.0 Intermediate 15-20 Susceptible≥21	Cerm +/-
1A	25	Susceptible	-
1B	0	Resistant	-
2B	6	Resistant	-
3A	0	Resistant	+
3B	0	Resistant	+
4B	13	Resistant	-
5A	13	Resistant	+
5B	0	Resistant	+
7B	20	Intermediate	-
9A	0	Resistant	-

9B	7	Resistant	-
9C	40	Susceptible	-
13A	24	Susceptible	-
13B	21	Susceptible	-
17A	7	Resistant	**+**
17B	11	Resistant	**+**
18A	11	Resistant	**+**
18B	23	Susceptible	**+**
21A	26	Susceptible	-
21B	9	Resistant	-
22A	30	Susceptible	**+**
25A	30	Susceptible	**+**
25B	29	Susceptible	**+**
28A	0	Resistant	**+**
28B	23	Susceptible	**+**
30A	26	Susceptible	**+**
30B	24	Susceptible	**+**
31A	0	Resistant	**+**
31B	9	Resistant	**+**
32A	44	Susceptible	-
32B	7	Resistant	-
34A	21	Susceptible	**+**
34B	16	Intermediate	**+**
36A	20	Intermediate	-
36B	26	Susceptible	-
37A	22	Susceptible	**+**
37B	16	Intermediate	**+**
39A	0	Resistant	-
39B	0	Resistant	-
42A	23	Susceptible	-
42B	22	Susceptible	-
44A	19	Intermediate	-
44B	30	Susceptible	-
44C	34	Susceptible	-
47A	26	Susceptible	-
47B	20	Intermediate	-
49A	26.5	Susceptible	**+**
49B	11	Resistant	**+**
50A	26	Susceptible	**+**
50B	0	Resistant	**+**
50C	25	Susceptible	**+**
53B	18	Intermediate	-
87B	38	Susceptible	-
101	40	Susceptible	-

	118A	25	Susceptible	-
	118B	30	Susceptible	-
	123A	19	Intermediate	+
	123B	0	Resistant	+
	125A	24	Susceptible	+
	125B	26	Susceptible	+

Table 5. Clindamycin ZOI and Resistance Genes. Classifications of susceptibility in comparison to the presence of the resistance gene, *Cerm(X)*.

The same 60 samples were also tested against tetracycline to determine susceptibility. Only seven on the samples were classified as resistant while none were deemed intermediate, leaving the remaining 53 samples to be classified as susceptible. When comparing the zones of inhibition to the molecular amplification of the resistance gene *tet(M)*, 28 samples were double confirmed while 32 were contradictory. The results are summarized in Table 6.

Sample	Zone of Inhibition (mm)	Resistant ≤14.0 Intermediate 15-18 Susceptible ≥ 19	Tet(M) +/-
1A	38	Susceptible	+
1B	13	Resistant	+
2B	39	Susceptible	-
3A	10	Resistant	+
3B	8	Resistant	+
4B	21	Susceptible	-
5A	21	Susceptible	-
5B	32	Susceptible	-
7B	32	Susceptible	-
9A	22	Susceptible	+
9B	23	Susceptible	+
9C	28	Susceptible	+
13A	27	Susceptible	+
13B	21	Susceptible	+
17A	42	Susceptible	-
17B	37	Susceptible	-
18A	38	Susceptible	+
18B	20	Susceptible	+
21A	22	Susceptible	+
21B	31	Susceptible	+

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22A	10	Resistant	+
25A	36	Susceptible	+
25B	31	Susceptible	+
28A	24	Susceptible	+
28B	32	Susceptible	+
30A	28	Susceptible	-
30B	27	Susceptible	-
31A	30	Susceptible	+
31B	30	Susceptible	+
32A	30	Susceptible	+
32B	34	Susceptible	+
34A	25	Susceptible	-
34B	28	Susceptible	-
36A	39	Susceptible	+
36B	36	Susceptible	+
37A	46	Susceptible	-
37B	32	Susceptible	-
39A	11	Resistant	-
39B	37	Susceptible	-
42A	27	Susceptible	+
42B	34	Susceptible	+
44A	36	Susceptible	-
44B	44	Susceptible	-
44C	26	Susceptible	-
47A	12	Resistant	+
47B	10	Resistant	+
49A	35	Susceptible	-
49B	36	Susceptible	-
50A	27	Susceptible	+
50B	22	Susceptible	+
50C	27	Susceptible	+
53B	35	Susceptible	-
87B	27	Susceptible	+
101	44	Susceptible	+
118A	34	Susceptible	-
118B	58	Susceptible	-
123A	25	Susceptible	+
123B	31	Susceptible	+
125A	32	Susceptible	+
125B	27	Susceptible	+

Table 6. Tetracycline ZOI and Resistance Genes. Classifications of susceptibility in comparison to presence of antibiotic resistance gene *tet(M)*.

Along with tetracycline and clindamycin, zones of inhibition were measured for rifampin and doxycycline and individual results are summarized in Appendix C. In Figure 1, samples were divided into three categories instead of four. Group zero consisted of samples taken from those who did not take antibiotics in the last two years, while group one is an antibiotic taken for either general illnesses or acne and group two is antibiotics taken for both general illnesses and acne. For each type of antibiotics, there is a slight decrease in the zone of inhibition in participants who have taken antibiotics for both acne and general illnesses in comparison with those who have not taken antibiotics in the past two years, with rifampin as the only exception. The results for rifampin were fairly similar among the different groups. Mean values for Tet0, Tet1 and Tet2 were 29.95, 29.65 and 28.27 millimeters respectively with a 1.68-millimeter decrease in diameter between TetO and Tet2. Mean values for Clinda0, Clinda1 and Clinda 2 were 22.21, 16.88 and 13.27 millimeters respectively with an 8.94-millimeter decrease in diameter between Clinda0 and Clinda2. Mean values for Rif0, Rif1 and Rif2 were 42.83, 44.94 and 43.93 millimeters respectively. The mean values for Doxy0, Doxy1 and Doxy2 were 33.57, 34.25 and 32.80 respectively with 0.77-millimeter decrease in diameter between Doxy0 and Doxy2. Standard deviations were also calculated.

Zones of Inhibition (mm)

60
50
40
30
20
10
0

TET 0 TET 1 TET 2 CLINDA 0 CLINDA 1 CLINDA 2 RIF 0 RIF 1 RIF 2 DOXY 0 DOXY 1 DOXY 2

Antibiotic discs and group comparisons
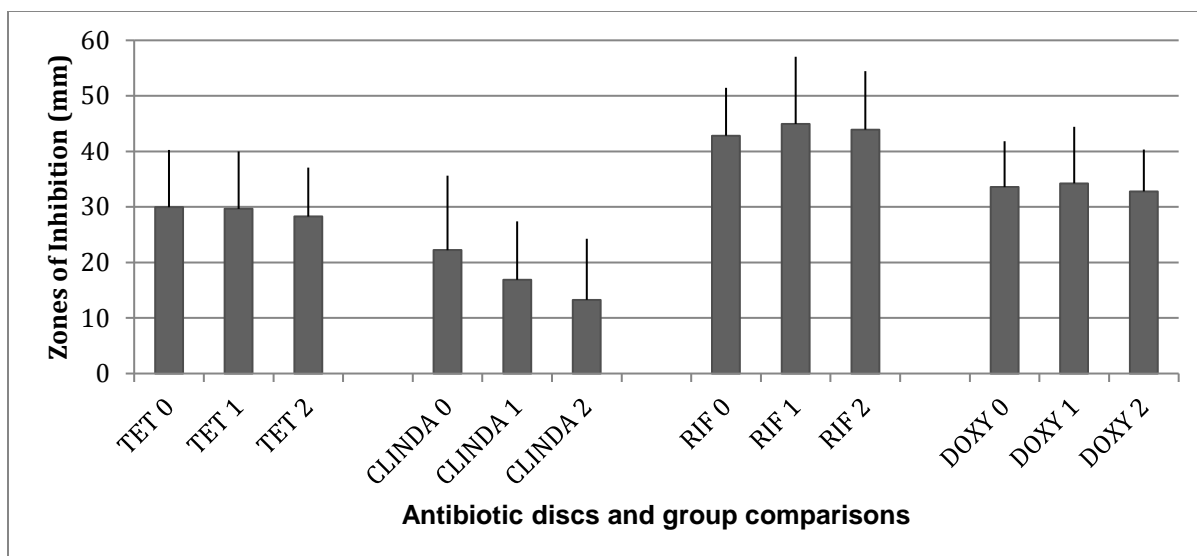
Figure 1. Comparison of mean zones of inhibition between categories in the first susceptibility testing (p<0.001). Participants are grouped similarly with the exception of category one and two, which were combined. P-value comparing Clinda0 and Clinda2 was 0.038 while comparison of Clinda0, 1 and 2 was 0.075.

Morphology Confirmation

In order to determine the zones of inhibition, each of these isolated samples were gram stained for morphology. A majority of the samples were gram positive, which were streaked onto Mannitol Salt agar (MSA) plates to check for a yellow color change. After salt preference was determined, samples were tested for catalase production utilizing hydrogen peroxide and the appearance of bubbles. A comprehensive list of microorganisms isolated can be found in Appendix C. Out of all isolated samples that were randomly tested for susceptibility, 44 were *Staphylococcus epidermidis* strains, while six were *Propionibacterium acnes* and six were *Staphylococcus aureus.* Three of the isolates were *Micrococcus spp,* with one *Enterococcus* isolate and two gram negative bacilli.

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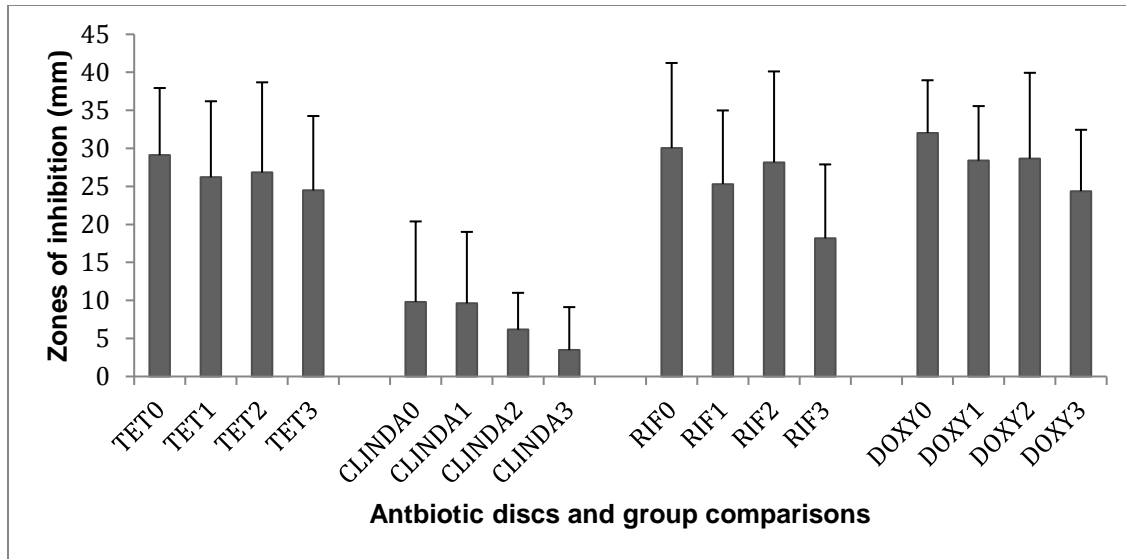
Figure 2. Comparison of mean zones of inhibition between categories in the second replication of susceptibility testing (p-value <0.001). Further post-hoc analysis determined the p-value resulting from the differences between Rif0 and Rif3 was 0.0015 while the p-value between Doxy0 and Doxy3 was 0.007.

Subsequent Susceptibility Testing

Another 42 samples were collected for a second round of susceptibility testing, following the procedure outline by BBL. Participants were grouped as previously outlined with group zero being no antibiotics in the last two years and group three being antibiotics for both acne and general illnesses. Group one participants have taken antibiotics only for generic illness while group two participants, specifically for acne. The same trend as previously observed could be inferred from Figure 2 where increased antibiotic use has a negative correlation with diameter of inhibition zones. The mean values for Tet0, Tet1, Tet2 and Tet3 where 29.11, 26.20, 26.83 and 24.47 millimeters respectively with a 4.64-millimeter decrease in diameter between Tet0 and Tet3. Mean values for Clinda0, Clinda1, Clinda2 and Clinda3 were 9.81, 9.61, 6.16 and 3.47

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respectively with a 6.34-millimeter decrease in diameter between Clinda0 and Clinda3. Mean values for Rif0, Rif1, Rif2 and Rif3 were 30.04, 25.31, 28.17 and 18.18 respectively with an 11.86-millimeter decrease in diameter between Rif0 and Rif3. Lastly, mean values for Doxy0, Doxy1, Doxy2 and Doxy3 were 32.04, 28.39, 28.67 and 24.38 respectively with a 7.66-millimeter decrease in diameter between Doxy0 and Doxy3. Total classifications of resistant, intermediate and susceptible samples are summarized in Table 7.

Antibiotic	Tetracycline	Clindamycin	Rifampin	Doxycycline
Resistant	15	75	24	1
Intermediate	14	18	9	3
Susceptible	82	19	79	107
Total Samples	111	112	112	111

Table 7. Comprehensive results of susceptibility testing

In Figure 3, samples were divided into antibiotic use or lack thereof, within the last two years. In tetracycline, there was a 3.22-millimeter decrease in the zone of inhibition in those who have taken antibiotics. In clindamycin, there was a 1.67-millimeter decrease in the zone of inhibition. In Figure 4, Rifampin diameter decreased 5.96 millimeters in those who had taken antibiotics while there was a 4.44-millimeter decrease in zones of inhibition around doxycycline discs.
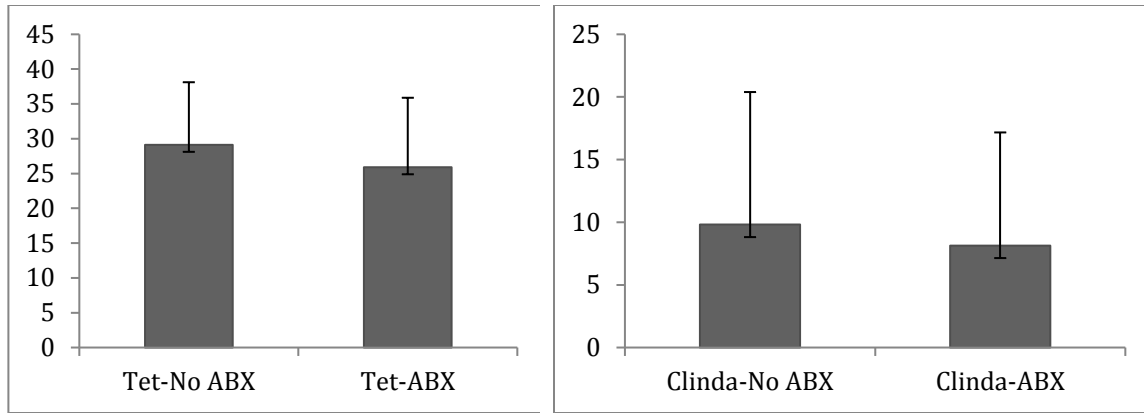
Figure 3. Comparison of Mean Diameters between participants. Zone of inhibition comparison between participants who have taken antibiotics and those who have not. The p-value between Tetracycline groups was 0.14 while the p-value for Clindamycin was 0.42.
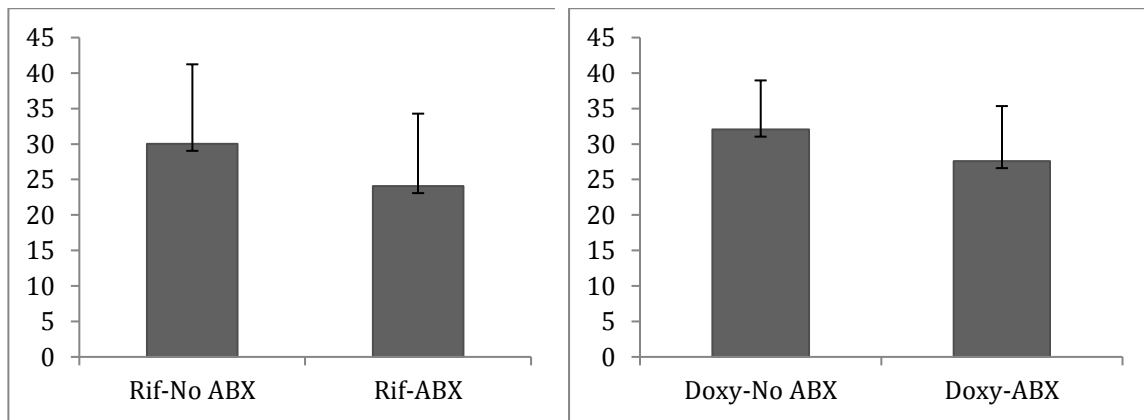
Figure 4. Comparison of Mean Diameters between participants. Zone of inhibition comparison between participants who have taken antibiotics and those who have not. The p-value between Rifampin groups was 0.009 while the p-value between Doxycycline groups was 0.012

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CHAPTER IV

DISCUSSION

The development of antibiotic resistance is a phenomenon that requires more attention than it has been given in the past. The current estimates suggesting devastating increases in resistance can no longer be ignored. Because the facial microbiota serve as a useful indicator of resistance development, it only made sense to utilize the bacteria found there. If current research can determine the presence of existing resistance genes as well as the current state of resistance cultures, more attention may be drawn to the problem at hand, equating to increased funding of pivotal research as well as increased production of new, more specific antibiotics.

Initial bacterial identification utilizing polymerase chain reaction established that a majority of samples contained *Propionibacterium acnes*, as 132 out of the total 144 samples were positive. *Staphylococcus epidermidis* was the second most common, with 110 positive samples while only 15 samples tested positive for *Staphylococcus aureus,* though previous research suggested that the figure should have been much greater. One particular explanation for a lack of positive *S. aureus* samples is too much primer, which has the potential to inhibit proper amplification of the bacterial DNA. Suggested final concentration of

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each primer in a reaction should be between 0.5-1mM and the final concentration utilized for this experiment was 1.95mM. It should be considered, however, that if inhibition was possible in *Staphylococcus aureus* samples, it is possible other bacterial prevalence would have increased also because the amount of primer added was consistent throughout. It is also important to note that microbial growth under aerobic conditions validated the lack of positive *Staphylococcus aureus* samples.

Resistance gene amplification samples were divided into groups based on consumption to determine if the presence of resistance genes demonstrated any correlation to antibiotic use. In group zero, specifying no antibiotics in the last two years, 22 of the 36 samples tested positive for *tet(M)* while 18 tested positive for *Cerm(X).* Group one, participants taking antibiotics for general illnesses, was composed of 74 samples, 51 testing positive for *tet(M)* and 31 testing positive for *Cerm(X)*. Group two, composed of six participants taking antibiotics solely for acne vulgaris, had five test positive for *tet(M)* and three for *Cerm(X)*. The remainder of the participants had taken antibiotics for both acne and general illnesses; 19 of the 28 samples tested positive for *tet(M)* and 10 tested positive for *Cerm(X)*. Half or more of each group tested positive for resistance to one or both of the resistance genes, even those who had not consumed antibiotics in the last two years. There was a slight increase in presence of *tet(M)* resistance between those who had not taken antibiotics and those who had taken at least two, though there was a decrease in prevalence of *Cerm(X)* between the groups. The prevalence of *tet(M)* resistance was greater than *Cerm(X)* across the board.

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Theoretically, there should have been a positive correlation between the prevalence of resistance genes and increased antibiotic consumption. One particular explanation for this phenomenon could be that a majority of those who had taken antibiotics had not taken macrolides or tetracycline. Another discrepancy lies in the reporting of antibiotic use of participants; many could not recall which antibiotic they had taken and some were unsure as to which prescriptions were classified as antibiotics.

Once resistance genes were amplified, susceptibility testing was performed. Sixty samples were tested using the first protocol outlined with 22 demonstrating resistance to clindamycin, eight intermediate and 30 were susceptible. Comparing these results to gene amplification, 34 results were double confirmed while 26 were contradictory. Of the sixty samples, only seven tested positive for resistance to tetracycline while the rest were susceptible and only 28 were double-confirmed by the presence of *tet(M)*. The samples used for susceptibility testing were grown overnight and measured after 48 hours. The guidelines in the BBL pamphlet recommend direct colony suspension for gram-positive samples while suggesting that overnight suspensions are not preferred. The samples should have been checked after 24 hours, instead of 48. In comparing the presence of resistance genes with the results of the susceptibility testing, the contradictions are not necessarily reliable because each sample used for molecular amplification were mixed cultures while the samples utilized for susceptibility were isolated colonies. Though some were susceptible, it is not entirely suggestive of susceptibility throughout, but individually. In order to test

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the entirety, one would have to isolate each colony present and test them. I isolated colonies based on control samples of *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis.* In each mixed sample, there may have been several strains of each bacterium, some of which carried the resistance gene amplified but was not the particular colony isolated.

It is also important to acknowledge the selective pressures that are forced upon bacteria by antibiotic use. Removing the bacteria from the environment that exposed them to such selective pressures may allow them to revert back to susceptible strains once allowed to multiply in incubation. The samples placed in PBS buffer were frozen and the environment leaves little to no room for replication. This phenomenon could help explain the discrepancy between the presences of resistance genes molecularly but demonstrated susceptibility when utilizing the Kirby Bauer method microbiologically.

Although the protocol from the initial 60 samples was not ideal, there was still a slight, if not great, decrease in the diameter of the zone of inhibition with greater consumption of antibiotics; rifampin was the only exception. This trend highlights the fact that increased antibiotic use leads to greater resistance or at least, decreased inhibition of the bacteria. With a p-value of <0.001, further analysis demonstrated that there was significant difference across the board with clindamycin use. The p-value was 0.038 when comparing no antibiotic use to double antibiotic use while the p-value was 0.075 comparing no antibiotic use against both one and two types of antibiotic use. Rifampin testing results were

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expected because there were no participants reporting the use of rifampin and previous research suggests no evidence of rifampin resistance in acne.

In order to confirm the decrease in zones of inhibition, a second round of susceptibility testing was performed utilizing the second protocol and direct colony suspensions. The trend was confirmed in greater magnitude. The lowest decrease in diameter with increased antibiotic consumption was 4.64 millimeters. The most surprising decrease was the 11.86-millimeter decrease when exposed to rifampin between those who had not taken antibiotics and those who had taken two types of antibiotics. The decrease is evident even between those who had not taken antibiotics in the last two years and those who had only used antibiotics for general illnesses. The p-value for the entire comparison was less than 0.001 and further analysis demonstrated that the difference between Rif0 and Rif3 was 0.0015 and 0.007 between Doxy0 and Doxy3. Dividing the samples into two groups based on antibiotic use or lack thereof, the decrease was still evident with a p-value of <0.01 between rifampin samples and 0.012 between doxycycline samples.

Though there were several significant differences and correlations that suggest increased antibiotic use causes a decrease in susceptibility to antibiotics, there are several ways to improve this thesis. If I were to restart this experiment, I would select a smaller sample size and would have isolated every single individual colony present and would have placed those immediately into PBS buffer and performed polymerase chain reaction on isolated rather than mixed cultures. I would have found Mueller-Hinton results for every single colony

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as well rather than a smaller subset of the initial population sampling. In subsequent experiments, I would do an initial measurement of the zone of inhibition and then, would have duplicated the experiment to test successive encounters with previously used antibiotics *in vitro*, utilizing the bacteria closest to the zone of inhibition. An interesting phenomenon that occurred frequently in this research was the presence of mutant colonies within the zones of inhibition on plates that had been exposed to rifampin. It would be beneficial to isolate those colonies and determine the resistance development occurring in successive encounters to the same antibiotic, especially considering previous research has not suggested significant rates of antibiotic resistance development while testing rifampin. Another trajectory that would be interesting for this research would be to utilize *Staphylococcus epidermidis* and its proven inhibition of *Propionibacterium acnes* in the battle against it.

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APPENDICES
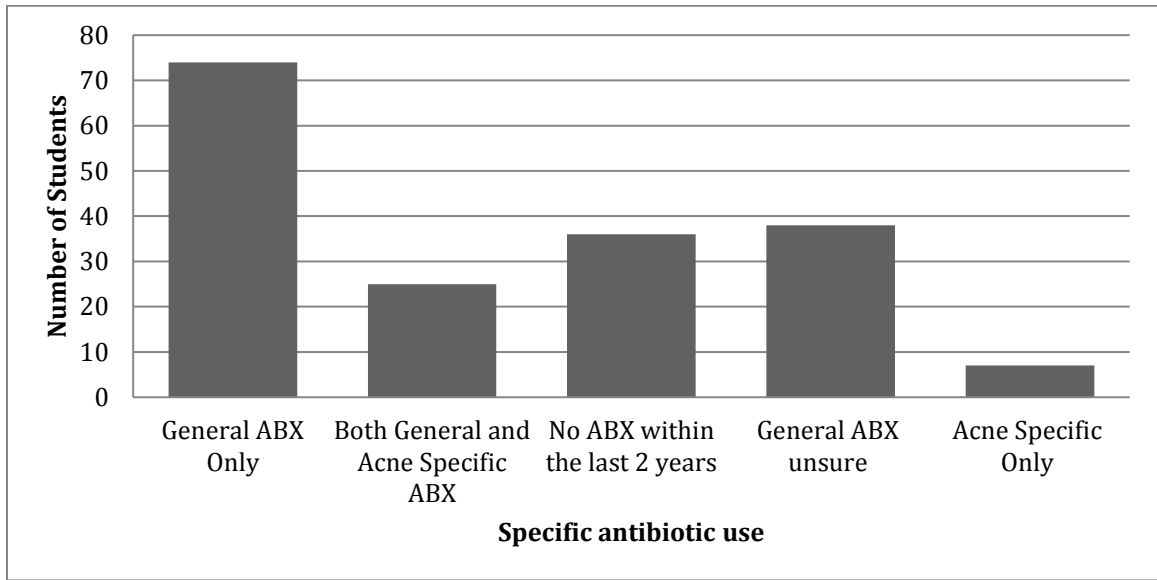
Appendix A: Initial Survey of Antibiotic Use
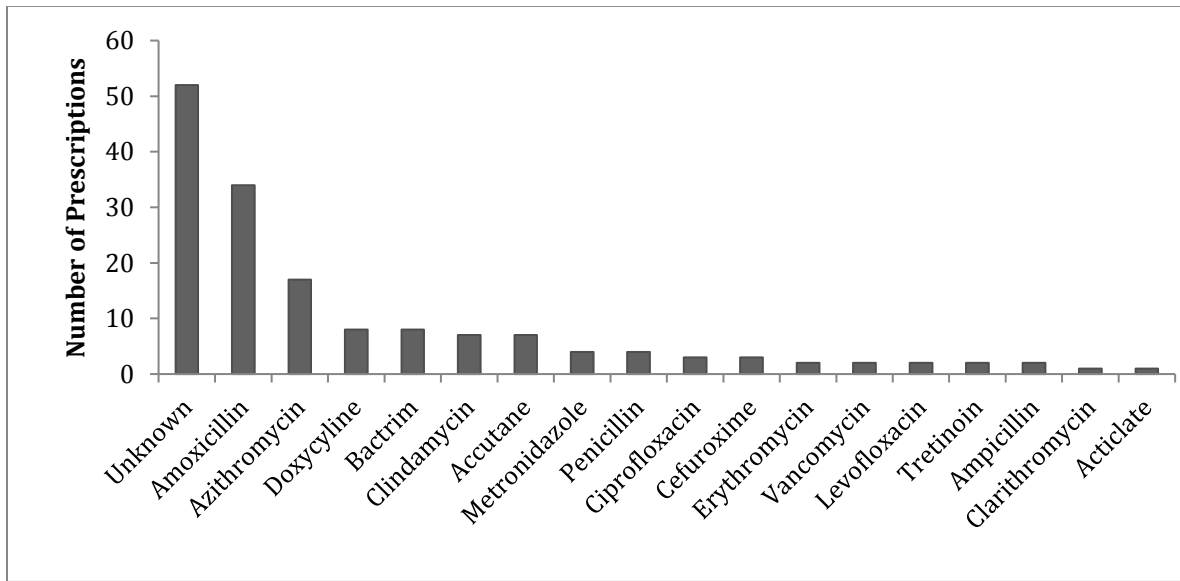
Appendix B: Initial Survey of Specific Antibiotics Prescribed.

Sample	Tetra (mm)	Clindamycin (mm)	Rifampin (mm)	Doxy (mm)	Gram +/-	Organism
1A	38	25	47	26	+	*Micrococcus*
1B	13	0	46	45	+	*S. epidermidis*
2A	42	13	59	36	-	
2B	39	6	31	22	+	*P. acnes*
3A	10	0	41	18	+	*S. epidermidis*
3B	8	0	49	20	+	*S. epidermidis*
4B	21	13	33	22	+	*S. epidermidis*
5A	21	13	21	31.5	+	*P. acnes*
5B	32	0	46	34	+	*S. epidermidis*
7B	32	20	40	37	+	*S. aureus*
9A	22	0	58	24	+	*S. epidermidis*
9B	23	7	54	24	+	*S. epidermidis*
9C	28	4	58	41	+	*S. epidermidis*
13A	27	24	42	29	+	*S. epidermidis*
13B	21	21	45	29	+	*S. epidermidis*
17A	42	7	44.5	43	+	*S. epidermidis*
17B	37	11	48	43	+	*S. epidermidis*
18A	38	11	21	40	+	*S. epidermidis*
18B	20	23	62	43	+	*S. epidermidis*
21A	22	26	54	24	+	*S. epidermidis*
21B	31	9	45	40	+	*P. acnes*
22A	10	30	36.5	34	+	*S. epidermidis*
22B	42	46	51	49	-	
25A	36	30	41	43	+	*S. aureus*
25B	31	29	46	27.5	+	*S. epidermidis*
28A	24	0	46.5	25	+	*S. epidermidis*
28B	32	23	40	31	+	*P. acnes*
30A	28	26	46	31	+	*S. epidermidis*
30B	27	24	33	30	+	*S. epidermidis*
31A	30	0	46	35	+	*Micrococcus*
31B	30	9	42	29	+	*S. epidermidis*
32A	30	44	49	32	+	*S. epidermidis*
32B	34	7	54	30	+	*S. epidermidis*
34A	25	21	11	30	+	*S. epidermidis*
34B	28	16	45	33	+	*S. epidermidis*
36A	39	20	52	42	+	*S. epidermidis*
36B	36	26	48	42	+	*S. epidermidis*
37A	46	22	64	48	+	*S. epidermidis*
37B	32	16	64	48	+	*S. epidermidis*
39A	11	0	58	23	+	*S. epidermidis*
39B	37	0	52	43	+	*S. epidermidis*
42A	27	23	43	30	+	*S. aureus*
42B	34	22	42	36	+	*S. epidermidis*

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44A	36	19	44	39	+	*S. epidermidis*
44B	44	30	44	45	+	*S. epidermidis*
44C	26	34	33	31	+	*Enterococcus*
47A	12	26	52	21	+	*S. epidermidis*
47B	10	20	42	16.5	+	*S. aureus*
49A	35	26.5	44	40	+	*S. epidermidis*
49B	36	11	24	36	+	*P. acnes*
50A	27	26	52	32	+	*S. epidermidis*
50B	22	0	36	24	+	*Micrococcus*
50C	27	25	48	28	+	*S. epidermidis*
53B	35	18	40	38.5	+	*S. epidermidis*
87B	27	38	35	39	+	*S. epidermidis*
101	44	40	61	46	+	*P. acnes*
118A	34	25	42	36	+	*S.aureus*
118B	58	30	38	60	+	*S. epidermidis*
123A	25	19	34	25.5	+	*S. epidermidis*
123B	31	0	35	31	+	*S. epidermidis*
125A	32	24	36	31	+	*S. epidermidis*
125B	27	26	32.5	25	+	*S. aureus*

Appendix C: Table of Susceptibility, Morphology and Species Identification

Presence and absence of organisms and resistance genes in those categorized in group 0: having taken no antibiotics within the last two years					
Sample	Tet(M)	Cerm(x)	*P. acnes*	*S. aureus*	S. epidermidis
2	-	-	+	-	+
3	+	+	+	-	+
6	-	-	+	-	+
16	+	+	+	-	+
21	+	-	+	-	+
22	+	+	+	-	+
25	+	+	+	-	+
30	-	+	+	-	+
32	+	-	+	-	+
38	+	+	+	-	+
40	+	+	+	-	+
44	-	+	+	-	+
45	+	+	+	-	+
48	+	-	-	-	+
53	-	-	+	+	-
54	-	-	+	-	+
58	-	-	+	+	-
59	+	+	+	-	+

Sample	Tet(M)	Cerm(X)	P. acnes	S. aureus	S. epidermidis
64	+	-	+	-	+
66	-	+	+	-	+
75	-	-	+	-	+
76	-	+	+	-	-
83	+	-	+	-	+
85	-	-	+	-	+
86	+	-	-	-	+
87	+	-	-	-	+
97	-	+	+	-	+
99	-	-	+	-	+
104	-	+	+	-	-
105	+	-	+	-	+
110	+	-	+	+	+
114	+	-	+	+	+
120	+	+	+	+	-
131	+	+	+	-	+
134	+	+	+	-	+
137	+	+	+	-	+

Appendix D: Results of Each Amplification in Group Zero

Presence and absence of organisms and resistance genes in those categorized in group 1: having taken antibiotics for general illnesses, not including acne					
Sample	Tet(M)	Cerm(X)	P. acnes	S. aureus	S. epidermidis
4	-	-	+	-	+
5	-	+	+	-	+
7	-	-	+	-	-
8	-	-	+	-	+
9	+	-	+	-	+
10	+	+	+	-	+
11	-	-	+	-	+
12	+	+	+	-	+
13	+	-	+	-	+
15	+	-	+	-	+
18	+	+	+	-	+
19	-	+	+	-	+
20	+	-	+	-	+
23	+	-	+	-	+
27	-	-	+	-	+
29	+	-	+	-	+
31	+	-	+	-	+
35	+	-	+	-	+

36	+	-	+	-	+
37	-	+	+	-	+
41	+	-	+	-	+
43	+	-	+	-	+
52	-	+	+	-	+
55	-	-	+	-	-
56	+	+	+	-	+
57	+	-	+	-	+
60	+	-	+	-	+
61	+	-	+	-	+
63	-	+	+	-	-
65	-	-	+	-	+
67	+	+	+	-	+`
69	+	-	+	-	+
70	+	+	+	-	-
71	-	+	+	-	+
72	+	-	+	-	+
73	+	+	+	-	-
77	-	-	+	-	-
79	+	-	-	-	-
80	-	-	-	-	+
81	+	+	-	-	+
84	+	+	+	-	+
88	-	-	+	+	+
89	-	-	+	-	+
90	+	-	+	-	-
93	+	+	+	-	+
94	-	-	+	-	-
95	+	+	+	-	+
98	+	-	+	-	+
100	+	-	-	-	-
101	+	-	+	+	+
102	+	-	+	-	+
108	+	+	+	-	+
111	+	+	+	+	+
113	-	+	+	-	+
115	+	+	+	+	+
116	+	+	-	+	-
117	+	-	+	-	+
118	-	-	-	-	-
119	+	-	+	+	+
121	-	+	+	-	+
123	+	+	+	-	+
124	-	-	+	-	-
126	-	+	+	-	-

55

128	+	+	+	-	+
129	+	-	+	-	+
133	+	+	+	-	+
135	+	+	+	-	+
136	+	+	+	-	+
138	+	+	+	-	+
139	+	-	+	-	-
140	+	-	+	-	+
142	+	-	+	-	+
143	+	+	+	-	+
144	+	-	+	-	+

Appendix E: Results of Each Amplification in Group One

Presence and absence of organisms and resistance genes in those categorized in group 2: those having taken antibiotics specifically for acne only					
Sample	Tet(M)	Cerm(X)	P. acnes	S. aureus	S. epidermidis
26	+	+	+	-	+
47	+	-	+	-	-
68	-	-	+	-	-
96	+	-	+	-	-
125	+	+	+	-	+
141	+	+	+	-	+

Appendix F: Results of each amplification designated to group two

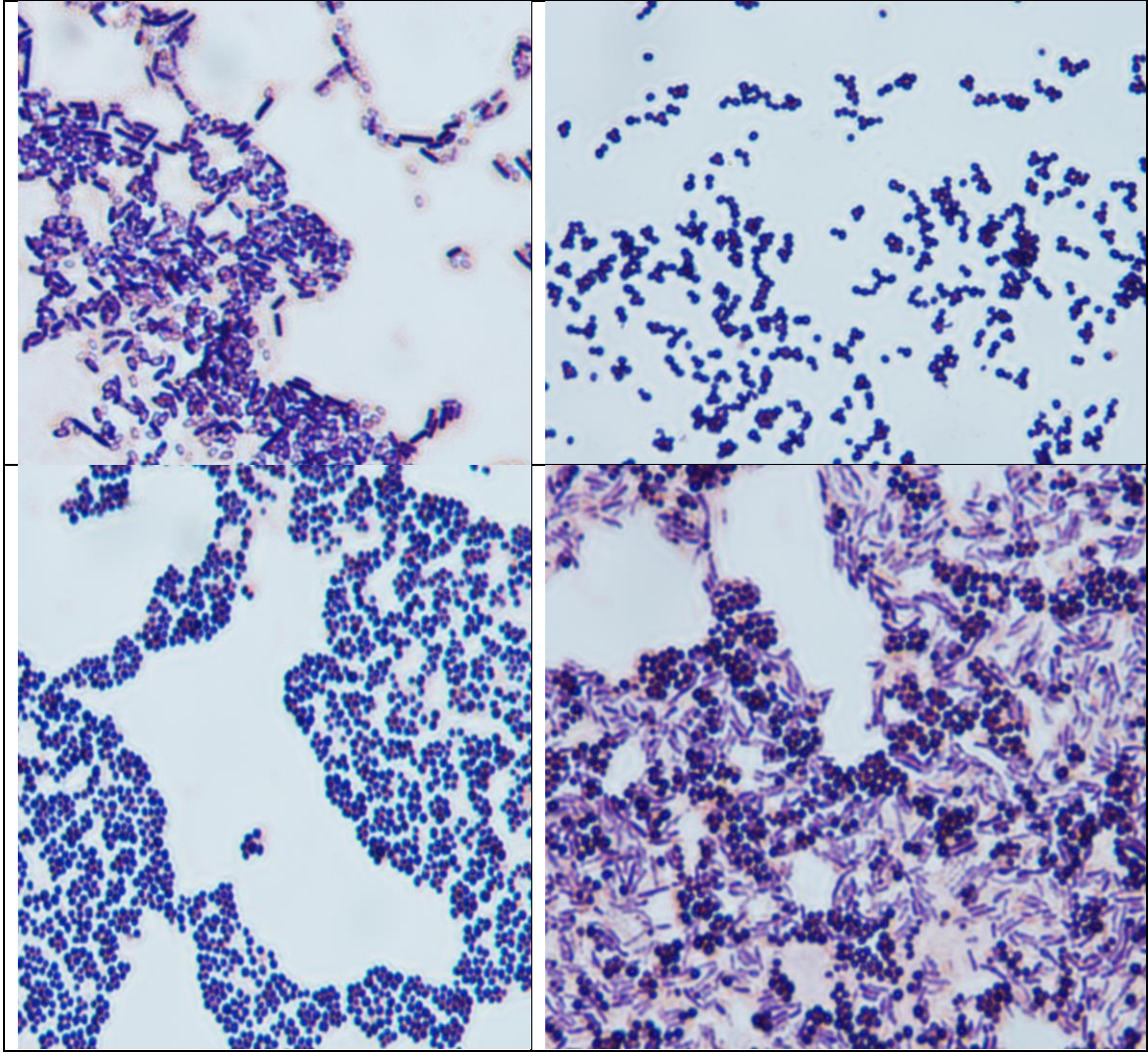| Presence and absence of organisms and resistance genes in those categorized in group 3: those having taken both antibiotics for general illnesses and acne					
Sample	Tet(M)	Cerm(X)	P. acnes	S. aureus	S. epidermidis
1	+	-	+	-	+
14	+	+	+	+	+
17	-	+	+	-	-
24	+	+	-	-	+

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28	+	+	+	-	+
33	-	-	+	-	-
34	-	+	+	-	+
39	-	-	+	-	-
42	-	-	+	-	+
46	+	-	+	-	+
49	-	+	+	-	+
50	+	+	+	+	-
51	+	-	+	-	+
62	+	-	-	-	-
74	+	-	+	-	-
78	-	-	+	-	-
82	+	+	+	-	+
91	+	-	+	+	+
92	-	-	+	-	-
103	+	-	+	-	-
106	+	-	+	-	+
107	+	-	+	-	+
109	-	-	-	+	-
112	+	-	+	-	-
122	+	+	+	-	+
127	+	-	+	-	+
130	+	-	+	-	-
132	+	+	+	-	+

**Appendix G: Results of Each Amplification in Group Three**

Appendix H: Common Morphologies-Gram Stains. Top left is a gram-positive bacilli (*Bacillus spp*), top right is gram-positive cocci (*S. epidermidis*), bottom left (*S. aureus*), and bottom right is common initial swab mixture.

Doxycycline & Rifampin ZOI in Gram Positive Organisms				
Sample	Doxycycline ZOI (mm)	R≤12.0 (I) 13-15 S ≥16	Rifampin ZOI (mm)	R≤16.0 (I) 17-19 S≥20
1A	26	S	47	S
1B	45	S	46	S
2B	22	S	31	S
3A	18	S	41	S
3B	20	S	49	S
4B	22	S	33	S
5A	31.5	S	21	S
5B	34	S	46	S
7B	37	S	40	S
9A	24	S	58	S
9B	24	S	54	S
9C	41	S	58	S
13A	29	S	42	S
13B	29	S	45	S
17A	43	S	44.5	S
17B	43	S	48	S
18A	40	S	21	S
18B	43	S	62	S
21A	24	S	54	S
21B	40	S	45	S
22A	34	S	36.5	S
25A	43	S	41	S
25B	27.5	S	46	S
28A	25	S	46.5	S
30A	31	S	46	S
30B	30	S	33	S
31A	35	S	46	S
31B	29	S	42	S
32A	32	S	49	S
32B	30	S	54	S
34A	30	S	11	R
34B	33	S	45	S
36A	42	S	52	S
36B	42	S	48	S
37A	48	S	64	S
37B	48	S	64	S
39A	23	S	58	S
39B	43	S	52	S
42A	30	S	43	S
42B	36	S	42	S

44A	39	S	44	S
44B	45	S	44	S
44C	31	S	33	S
47A	21	S	52	S
47B	16.5	S	42	S
49A	40	S	44	S
49B	36	S	24	S
50A	32	S	52	S
50B	24	S	36	S
50C	28	S	48	S
53B	38.5	S	40	S
87B	39	S	35	S
101	46	S	61	S
118A	36	S	42	S
118B	60	S	38	S
118B	60	S	38	S
123A	25.5	S	34	S
123B	31	S	35	S
125A	31	S	36	S
125B	25	S	32.5	S

Appendix I: Comprehensive Results for Doxycycline and Rifampin
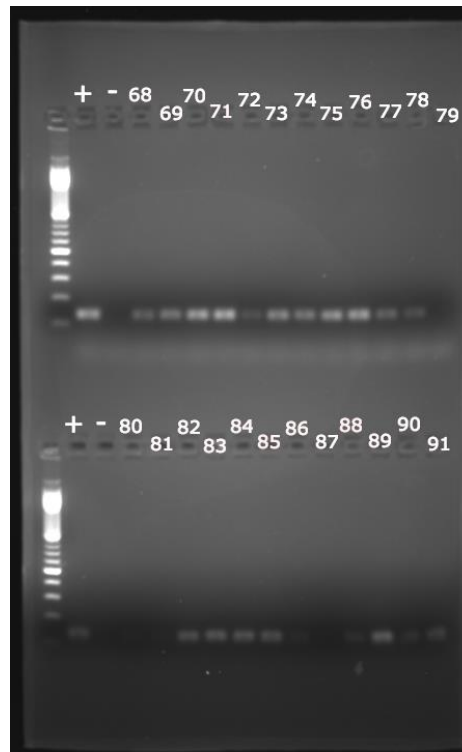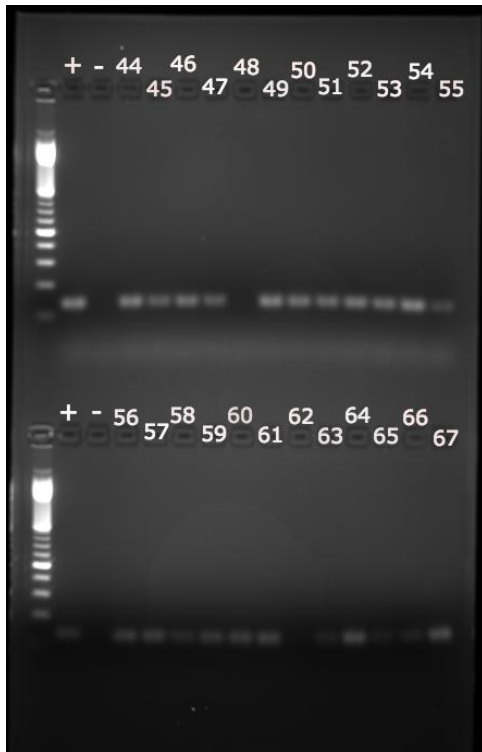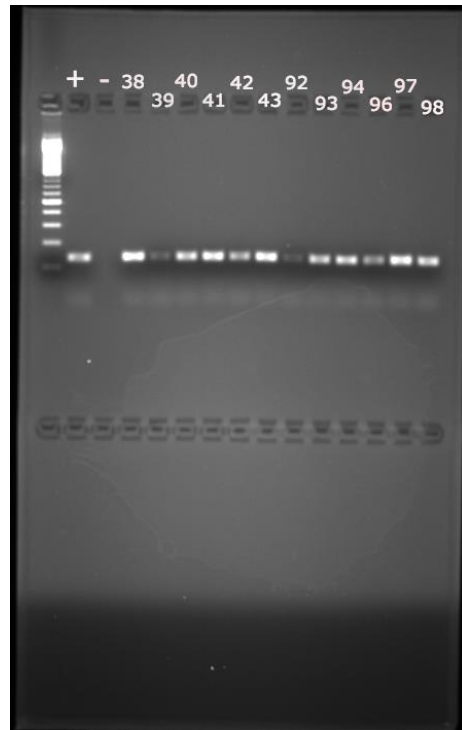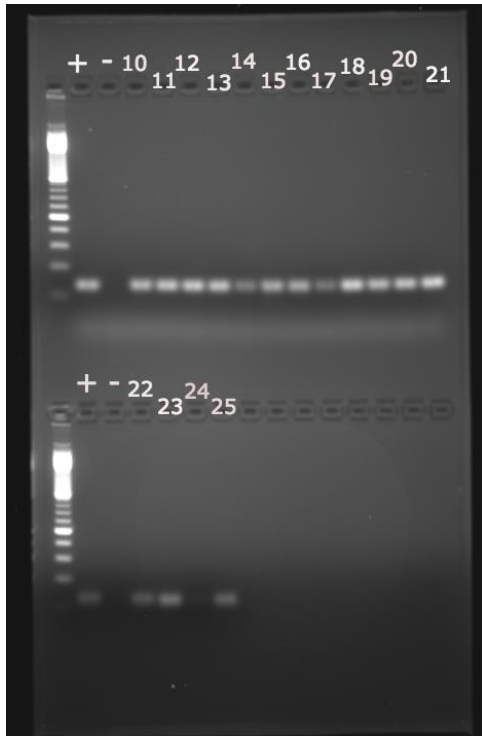
Appendix J: P acne primer samples. Top left are samples 10-21, top right are samples 38-43 and 92-98, bottom right are samples 44-67 and bottom left is 68-91.
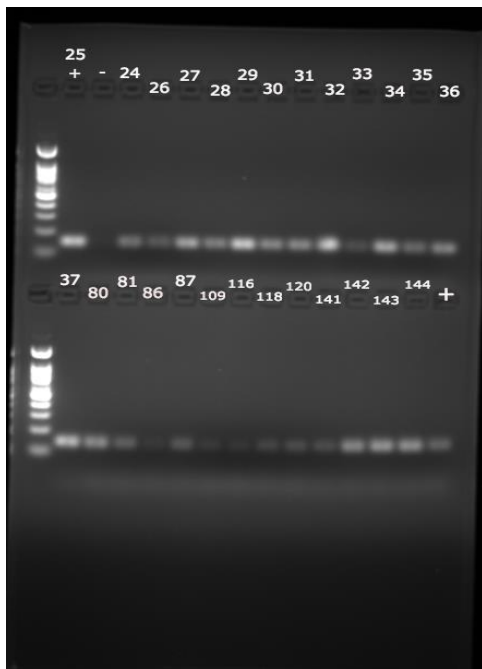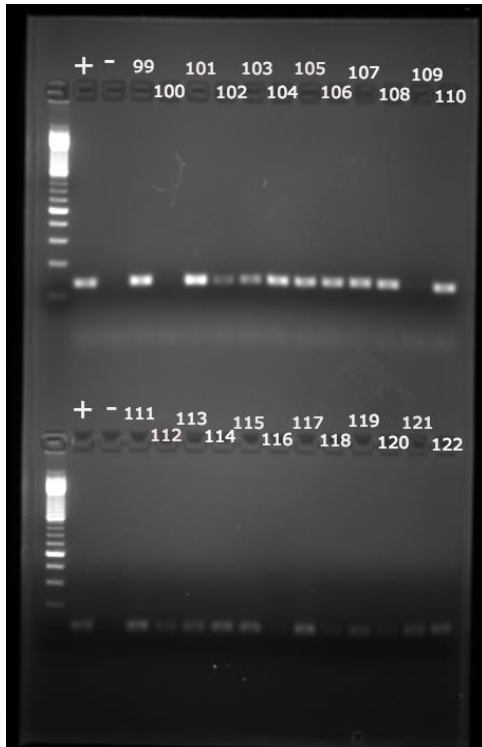
Appendix K: P. acne primer samples. Samples 99-122, 123-140, 2-9 and repeats for double confirmation and samples 141-144.
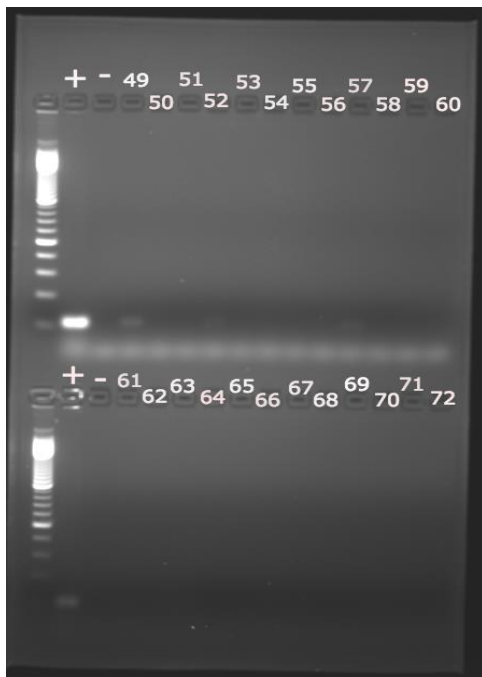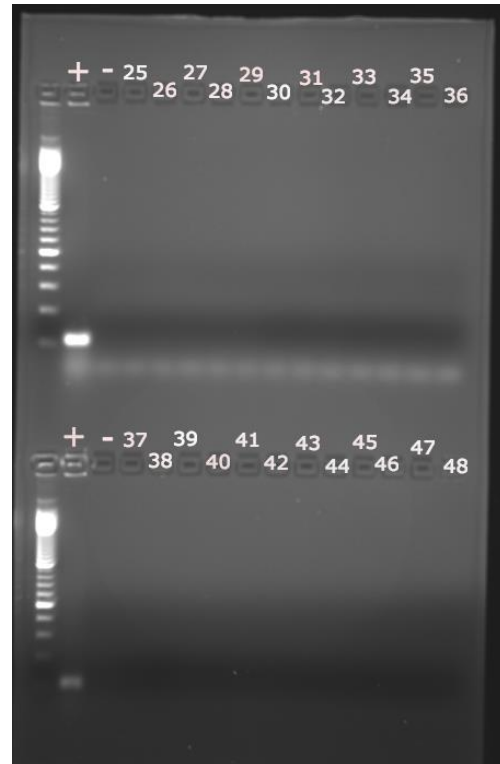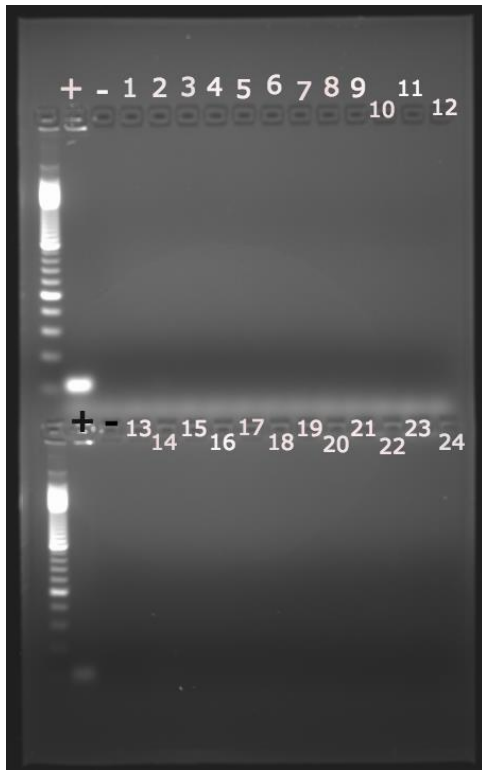
Appendix L: S. aureus primers. Top left are samples 1-24, top right is samples 25-48, bottom left are samples 49-72 and bottom right are samples 73-96.
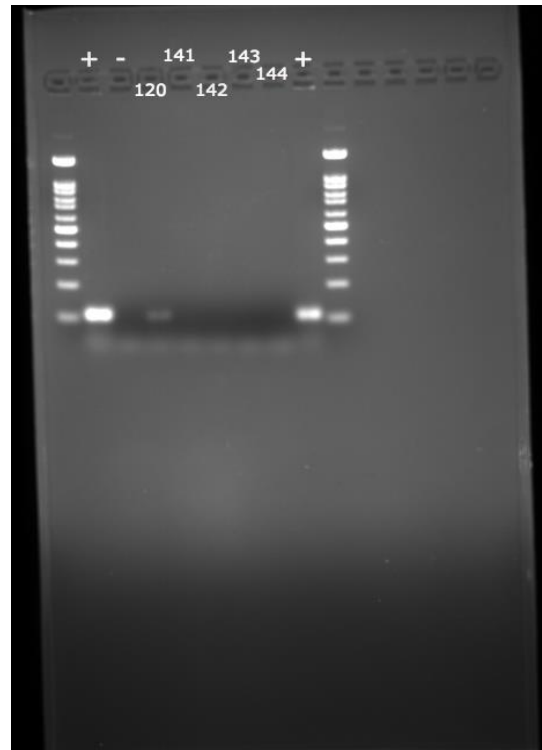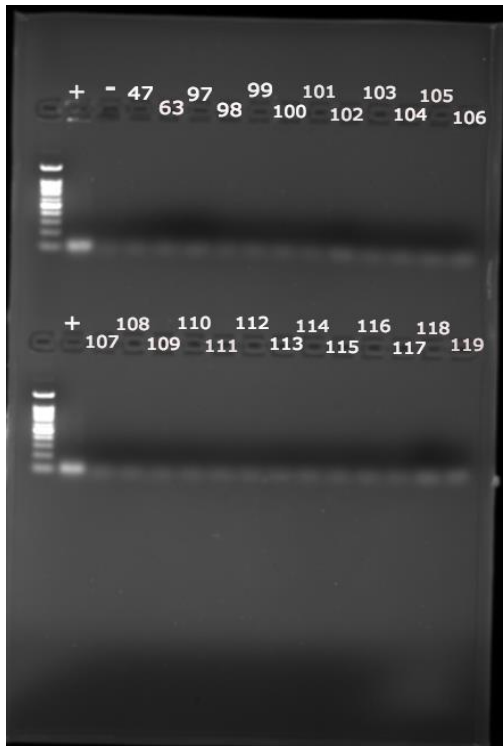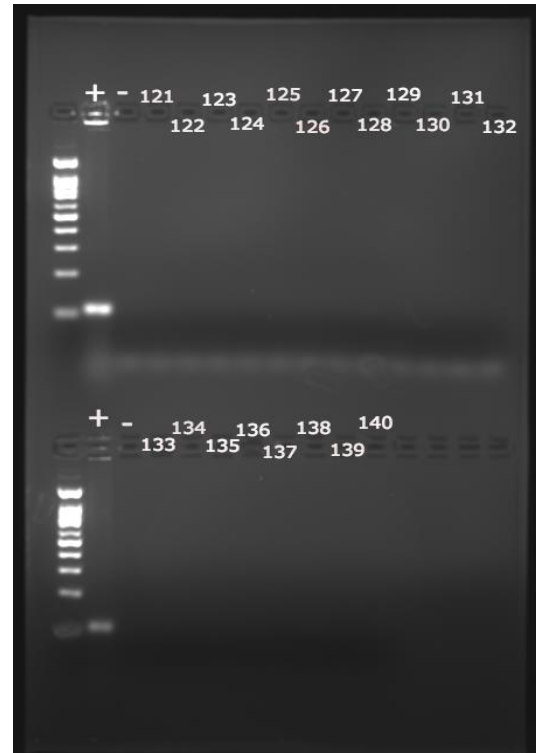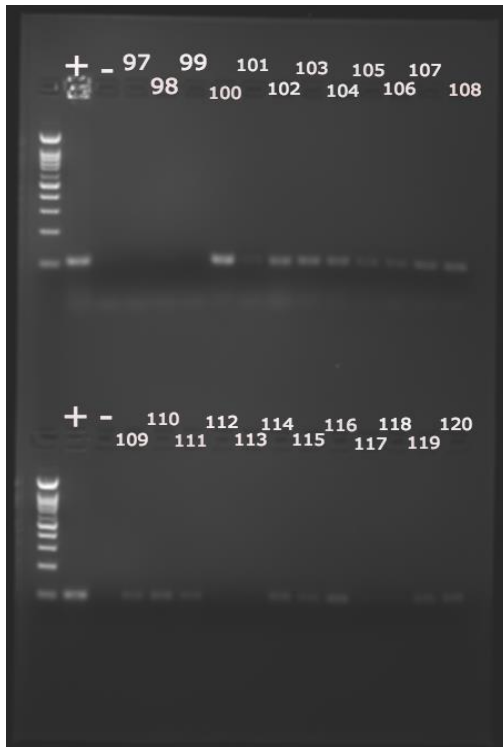
Appendix M: S. aureus primers. Top left are samples 97-120, top right are samples 121-140. Bottom left are sample repeats for confirmation and right are samples 120, 141-144.
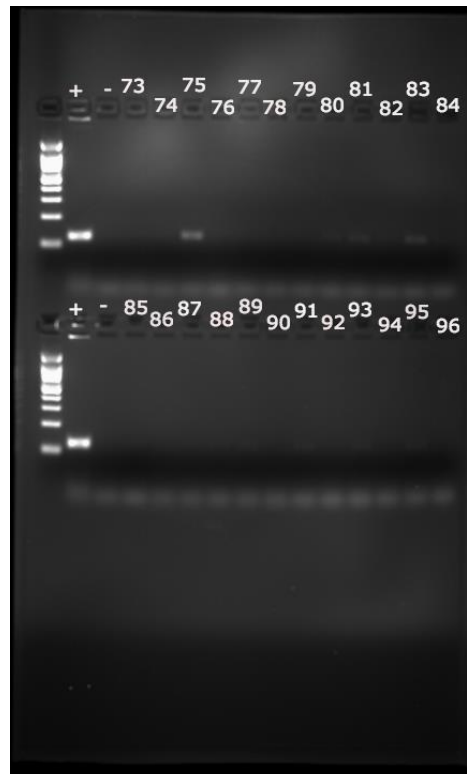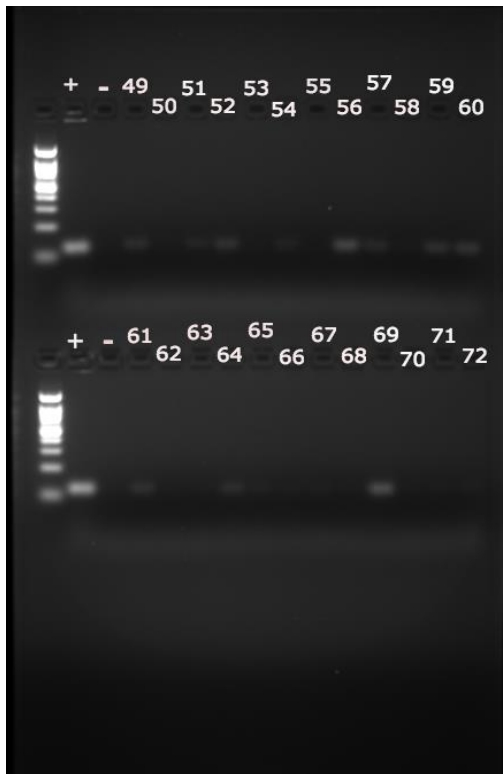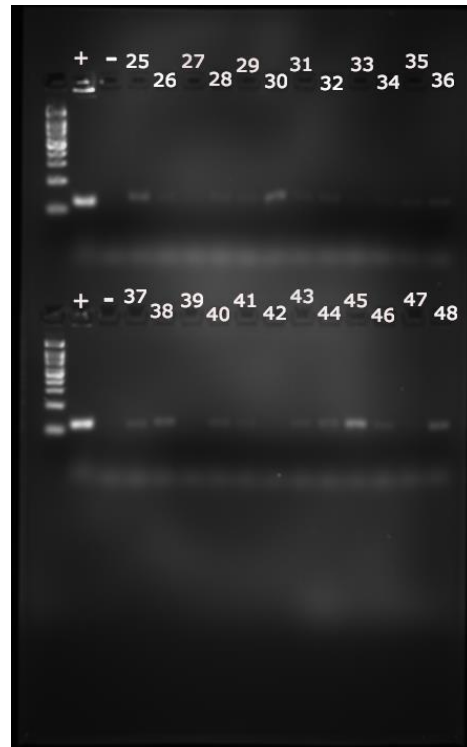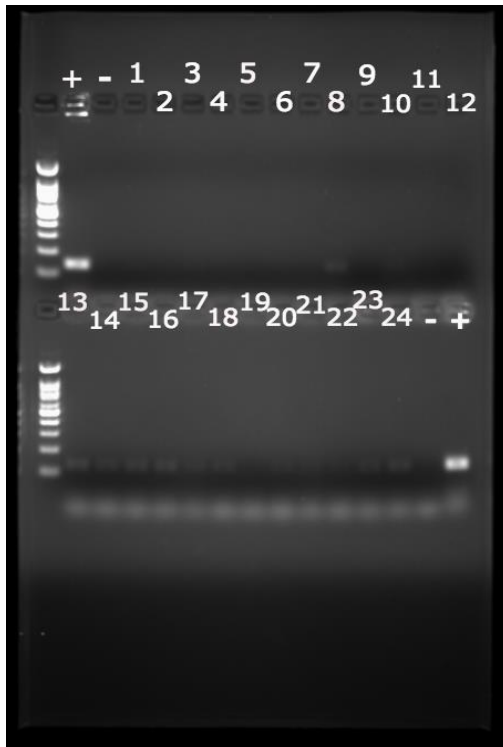
Appendix N: S. epidermidis primers. Top left are samples 1-24 and top right are samples 25-48. Bottom left are samples 49-72 and right are samples 73-96.
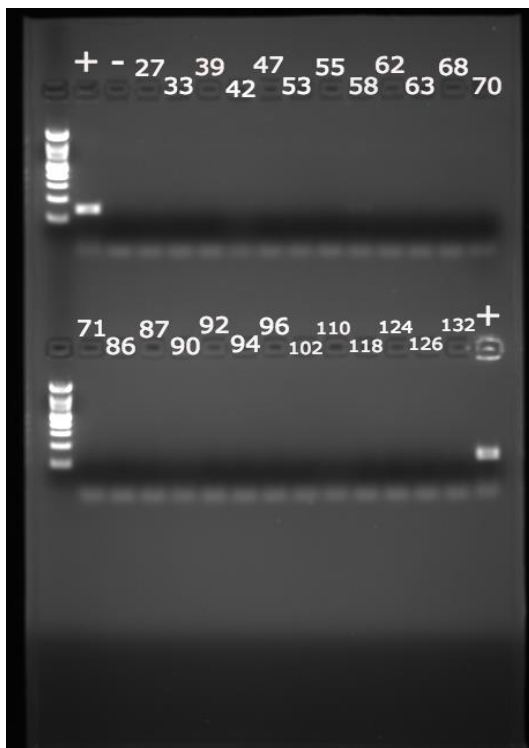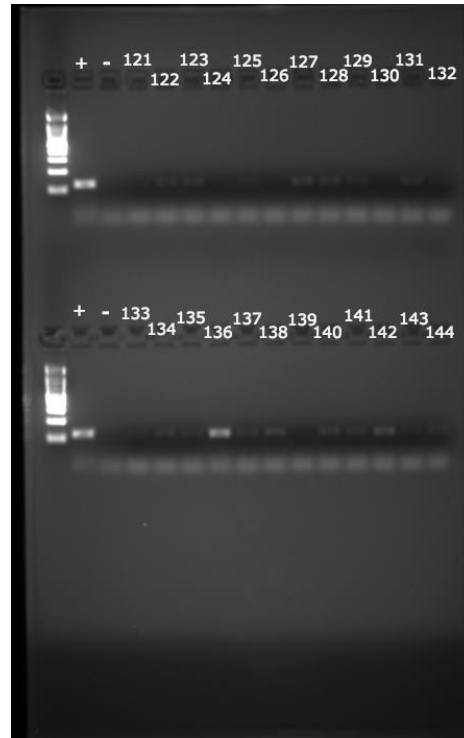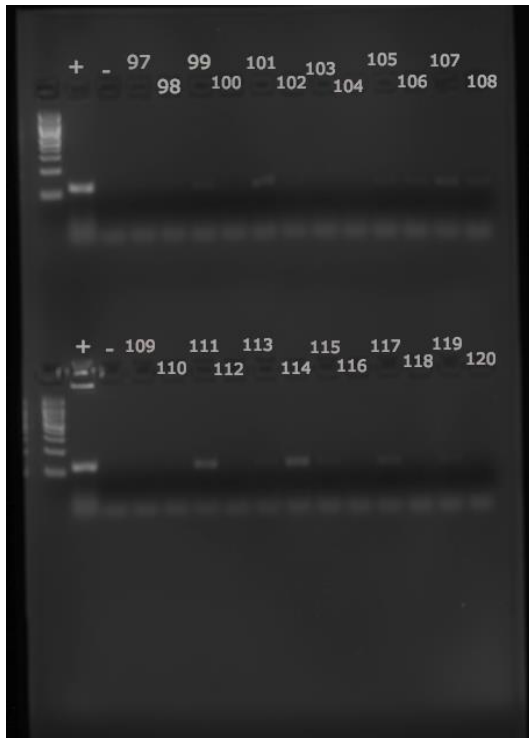
Appendix O: S. epidermidis samples. Top left are samples 97-120 and top right are samples 121-144. Bottom left are repeats for confirmation.

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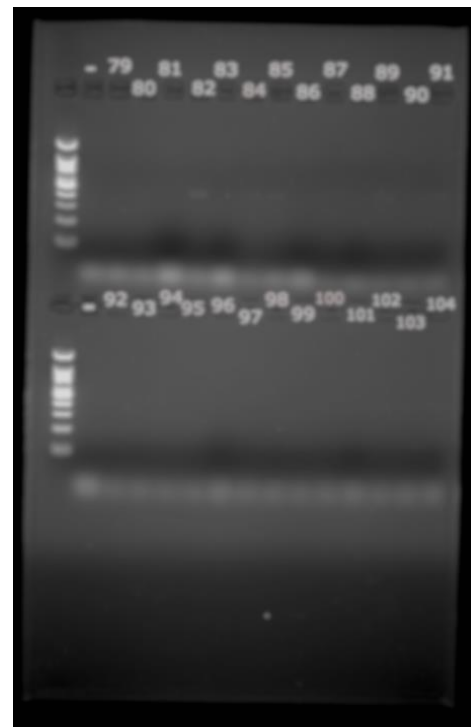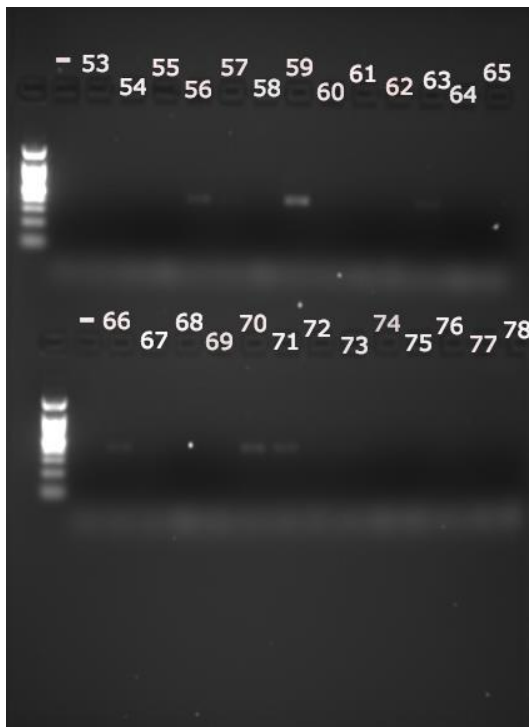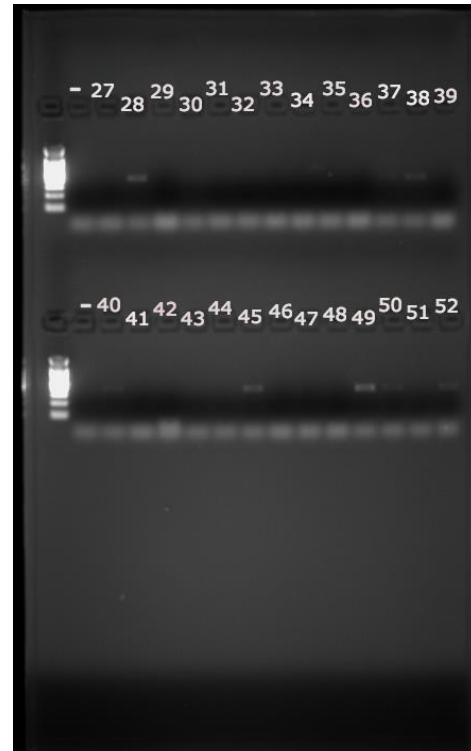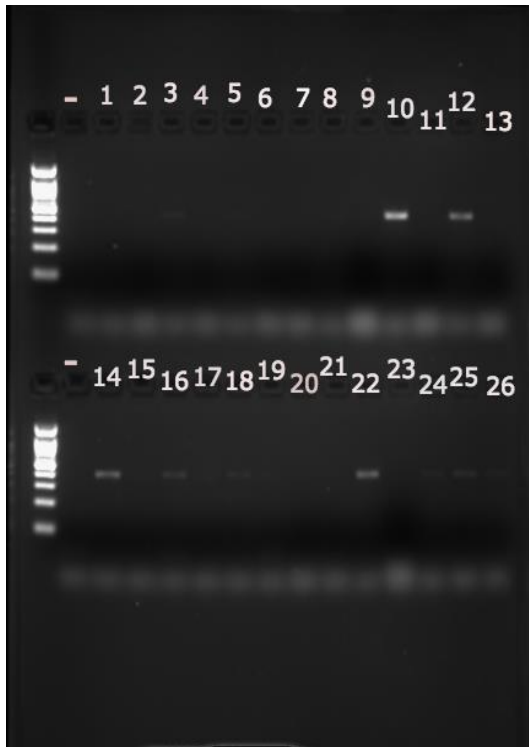
Appendix P: Cerm(X) resistance primers. Top left are samples 1-26, top right are samples 27-52, bottom left are samples 53-78 and bottom right are samples 79-104.
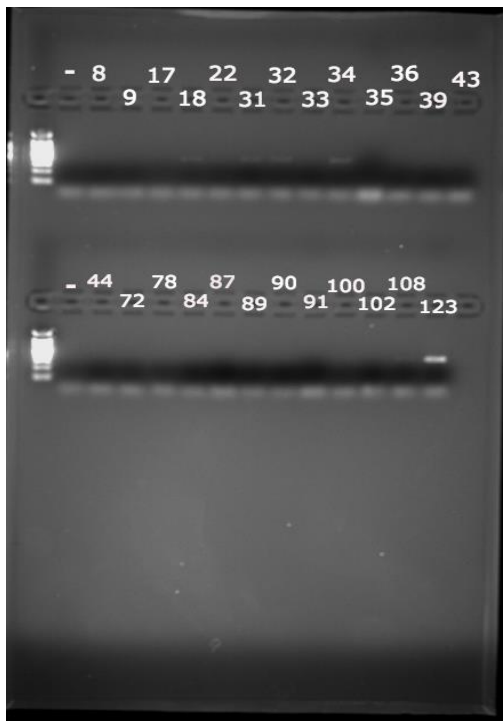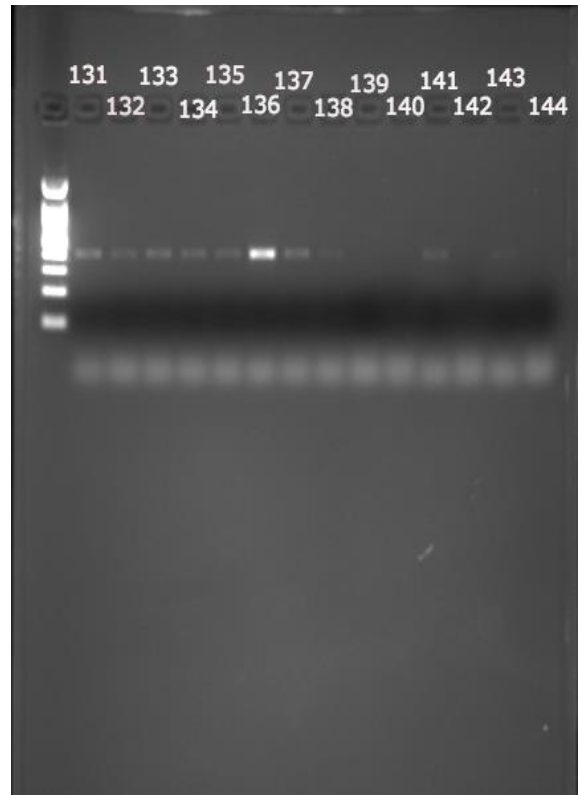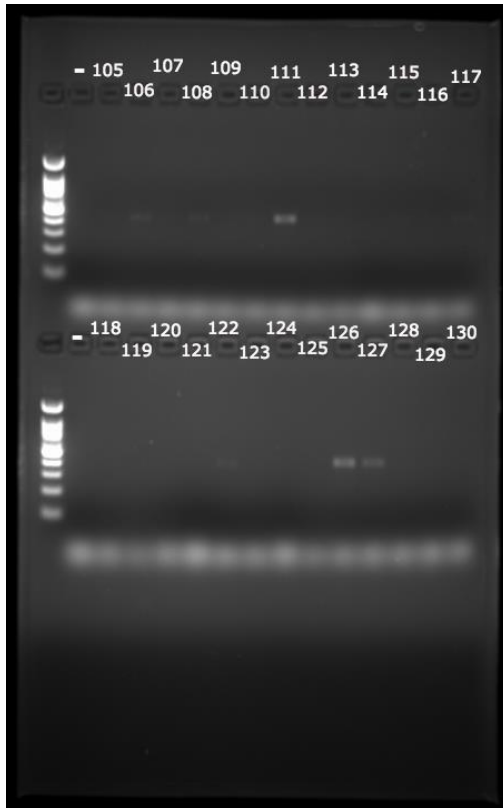
Appendix Q: Cerm(X) resistance primers. Top left are samples 105-130, top right are samples 131-144. Bottom left are repeats for double confirmation.
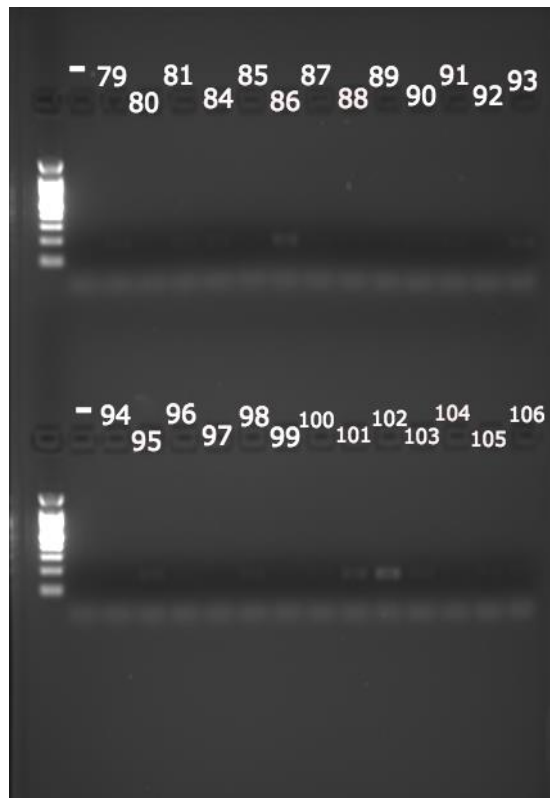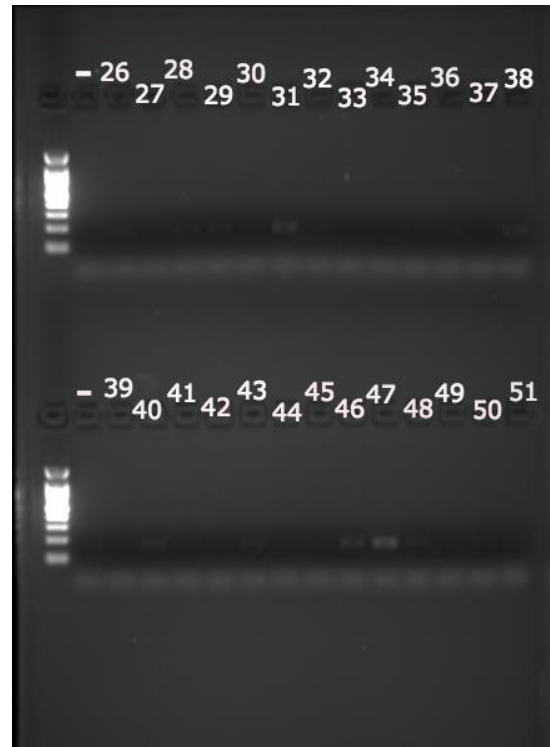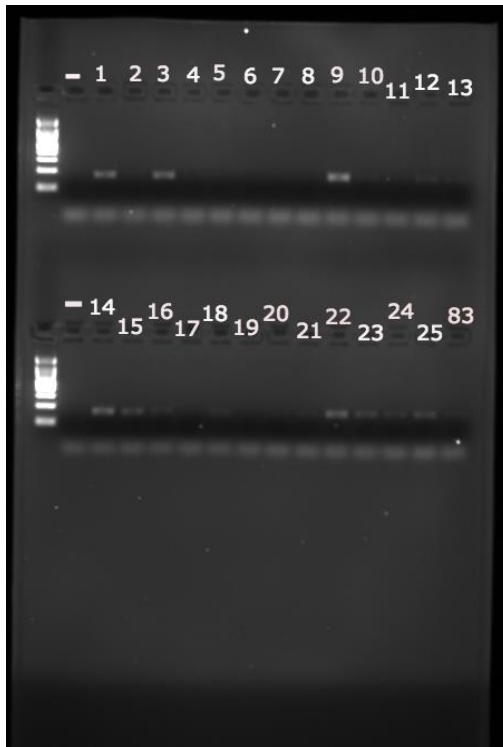
Appendix R: tetM resistance primers. Top left are samples 1-25 and 83. Top right are samples 26-51. Bottom left are samples 52-78 and 82. Bottom right are samples 79-106.
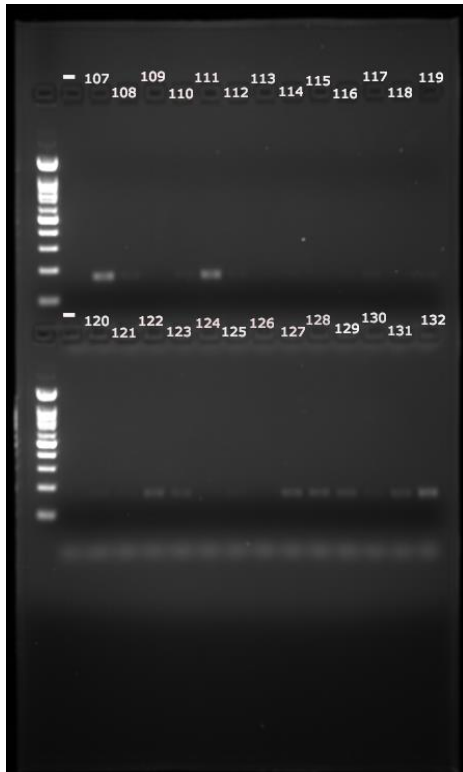
Appendix S: tet(M) resistance primers. Top left are samples 107-132. Top right are samples 133-144. Bottom left are sample repeats for double confirmation.

BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs

			Zone Diameter Interpretive Chart †									
			Zone Diameter Interpretive Standards (mm)			Control Zone Diameter Limits (mm)						
Antimicrobial Agent	Code	Disc Potency	Resis-tant	Inter-mediate[a]	Suscep-tible[b]	E. coli ATCC 25922	S. aureus ATCC 25923	P. aeruginosa ATCC 27853	H. influenzae ATCC 49247[c]	H. influenzae ATCC 49766[c]	N. gonorrhoeae ATCC 49226[d]	S. pneumoniae ATCC 49619[e]
Ciprofloxacin	CIP-5	5 µg				30 – 40	22 – 30	25 – 33				
Enterobacteriaceae,[ddd] P. aeruginosa, Acinetobacter, staphylococci and enterococci			≤15	16 – 20	≥21							
Haemophilus spp. [c]			—	—	≥21				34 – 42[c]	—		
N. gonorrhoeae [d]			≤27	28 – 40[w]	≥41						48 – 58[d]	
Doxycycline [ee]	D-30	30 µg				18 – 24	23 – 29	—				
Enterobacteriaceae[aa], P. aeruginosa, Acinetobacter[aa], staphylococci and enterococci [yy]			≤12	13 – 15	≥16							
Rifampin	RA-5	5 µg				8 – 10	26 – 34	—				
Staphylococcus spp. and Enterococcus spp. [yy]			≤16	17 – 19	≥20							
Haemophilus spp. [c]			≤16	17 – 19	≥20				22 – 30[c]	—		
S. pneumoniae [e]			≤16	17 – 18	≥19							25 – 30[e]
Tetracycline [ee]	Te-30	30 µg				18 – 25	24 – 30	—				
Enterobacteriaceae[aa], P. aeruginosa, Acinetobacter[aa], staphylococci, enterococci [yy] and V. cholerae [m]			≤14	15 – 18	≥19							
Haemophilus spp. [c]			≤25	26 – 28	≥29				14 – 22[c]	—		
N. gonorrhoeae [d,uu]			≤30	31 – 37[w]	≥38						30 – 42[d]	
S. pneumoniae and other streptococci [e]			≤18	19 – 22	≥23							27 – 31[e]

Appendix T: BD BBL Sensi-Disc Susceptibility Chart

Tetracycline Resistance				Clindamycin Resistance			
Sample	Zone of Inhibition	R ≤14 S ≥19	Category	Sample	ZOI	R ≤ 14 S ≥ 21	Category
145A	29	S	3	145A	21	S	3
146A	11	R	3	146A	0	R	3
146B	18	I	3	146B	0	R	3
146C	20	S	3	146C	0	R	3
146M	17	I	3	146M	0	R	3
154A	27	S	3	154A	0	R	3
154B	42	S	3	154B	0	R	3
154C	43	S	3	154C	0	R	3
154M	11	R	3	154M	0	R	3
166A	19	S	3	166A	20	S	3
166B	20	S	3	166B	0	R	3
166M	18	I	3	166M	7	R	3
167A	34	S	3	167A	0	R	3
167M	18	I	3	167M	0	R	3
168A	34	S	3	168A	11	R	3
168B	32	S	3	168B	0	R	3
168M	23	S	3	168M	0	R	3
163A	20	S	2	163A	10	R	2
163B	24	S	2	163B	0	R	2
163M	26	S	2	163M	0	R	2
165A	45	S	2	165A	10	R	2
165B	35	S	2	165B	9	R	2
165M	11	R	2	165M	8	R	2
149A	37	S	1	149A	20	I	1
149B	40	S	1	149B	11	R	1
149M	30	S	1	149M	17	I	1
150A	24	S	1	150A	0	R	1
150B	26	S	1	150B	0	R	1
150M	0	R	1	150M	0	R	1
151A	41	S	1	151A	0	R	1
151B	34	S	1	151B	19	I	1
151M	32	S	1	151M	20	I	1
152A	25	S	1	152A	17	I	1
152B	40	S	1	152B	0	R	1
152M	24	S	1	152M	23	S	1
153A	23	S	1	153A	0	R	1
153B	41	S	1	153B	0	R	1
153M	14	R	1	153M	8	R	1

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155A	35	R	1	155A	14	R	1
155M	28	R	1	155M	17	I	1
156A	17	I	1	156A	0	R	1
156M	21	S	1	156M	17	I	1
157A	35	S	1	157A	0	R	1
157M	12	R	1	157M	12	R	1
158A	26	S	1	158A	0	R	1
158B	43	S	1	158B	0	R	1
158C	39	S	1	158C	9	R	1
158M	26	S	1	158M	0	R	1
161A	40	S	1	161A	0	R	1
161M	23	S	1	161M	0	R	1
162A	34	S	1	162A	0	R	1
162M	13	R	1	162M	17	I	1
169A	30	S	1	169A	26	S	1
169M	12	R	1	169M	7	R	1
170A	34	S	1	170A	11	R	1
170B	8	R	1	170B	11	R	1
170M	13	R	1	170M	13	R	1
171A	34	S	1	171A	17	I	1
171M	28	S	1	171M	11	R	1
172A	34	S	1	172A	0	R	1
172B	18	I	1	172B	21	S	1
172C	17	I	1	172C	15	I	1
172M	11	R	1	172M	0	R	1
173A	29	S	1	173A	21	S	1
173B	11	R	1	173B	0	R	1
173M	24	S	1	173M	9	R	1
176A	31	S	1	176A	28	S	1
176M	28	S	1	176M	0	R	1
180A	40	S	1	180A	0	R	1
180M	32	S	1	180M	0	R	1
181A	29	S	1	181A	26	S	1
181M	13	R	1	181M	0	R	1
182A	14	R	1	182A	18	I	1
182B	36	S	1	182B	0	R	1
182M	34	S	1	182M	0	R	1
183A	20	S	1	183A	25	S	1
183M	16	I	1	183M	18	I	1
184A	30	S	1	184A	24	S	1
184M	15	I	1	184M	20	I	1

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185A	29	S	1	185A	0	R	1
185B	29	S	1	185B	15	I	1
185C	22	S	1	185C	22	S	1
185M	18	I	1	185M	2	R	1
186A	36	S	1	186A	15	I	1
147A	29	S	0	186M	0	R	1
147B	41	S	0	147A	24	S	0
147M	31	S	0	147B	24	S	0
148A	35	S	0	147M	24	S	0
148B	35	S	0	148A	0	R	0
148M	44	S	0	148B	22	S	0
159A	34	S	0	148M	23	S	0
159M	21	S	0	159A	0	R	0
160A	36	S	0	159M	8	R	0
160M	23	S	0	160A	0	R	0
164A	37	S	0	160M	16	I	0
164B	15	I	0	164A	0	R	0
164M	16	I	0	164B	0	R	0
174A	35	S	0	164M	0	R	0
174B	18	I	0	174A	22	S	0
174C	36	S	0	174B	0	R	0
174M	28	S	0	174C	23	S	0
175A	24	S	0	174M	18	I	0
175M	17	I	0	175A	0	R	0
177A	31	S	0	175M	0	R	0
177M	25	S	0	177A	0	R	0
178A	38	S	0	177M	18	I	0
178M	19	S	0	178A	0	R	0
179A	31	S	0	178M	10	R	0
179B	31	S	0	179A	8	R	0
179C	42	S	0	179B	25	S	0
179M	14	R	0	179C	0	R	0
				179M	0	R	0

Appendix U: Tetracycline and Clindamycin ZOI Organized by Category

Rifampin Resistance				Doxycycline Resistance			
Sample	ZOI	R ≤ 16 S ≥ 20	Category	Sample	ZOI	R ≤ 12 S ≥ 16	Category
145A	15	R	3	145A	30.5	S	3
146A	45	S	3	146A	25	S	3
146B	8	R	3	146B	17	S	3
146C	9	R	3	146C	17	S	3
146M	8	R	3	146M	14	I	3
154A	11	R	3	154A	26	S	3
154B	25	S	3	154B	40	S	3
154C	24	S	3	154C	39	S	3
154M	22	S	3	154M	17	S	3
166A	20	S	3	166A	26	S	3
166B	9	R	3	166B	18	S	3
166M	10	R	3	166M	19	S	3
167A	26	S	3	167A	30	S	3
167M	24	S	3	167M	22	S	3
168A	20	S	3	168A	35	S	3
168B	23	S	3	168B	20	S	3
168M	10	R	3	168M	19	S	3
163A	37	S	2	163A	30	S	2
163B	24	S	2	163B	33	S	2
163M	21	S	2	163M	24	S	2
165A	42	S	2	165A	42	S	2
165B	35	S	2	165B	34	S	2
165M	10	R	2	165M	9	R	2
149A	32	S	1	149A	40	S	1
149B	36	S	1	149B	40	S	1
149M	41	S	1	149M	27	S	1
150A	26	S	1	150A	33	S	1
150B	25	S	1	150B	33	S	1
150M	41	S	1	150M	18	S	1
151A	24	S	1	151A	38	S	1
151B	18	I	1	151B	35	S	1
151M	16	R	1	151M	31	S	1
152A	19	I	1	152A	28	S	1
152B	25	S	1	152B	38	S	1
152M	12	R	1	152M	26	S	1
153A	18	I	1	153A	28	S	1
153B	19	I	1	153B	37	S	1
153M	39	S	1	153M	19	S	1

155A	32	S	1	155A	34	S	1
155M	32	S	1	155M	24	S	1
156A	24	S	1	156A	18	S	1
156M	18	I	1	156M	31	S	1
157A	8	R	1	157A	36	S	1
157M	15	R	1	157M	16	S	1
158A	12	R	1	158A	24	S	1
158B	22	S	1	158B	41	S	1
158C	24	S	1	158C	38	S	1
158M	34	S	1	158M	27	S	1
161A	15	R	1	161A	42	S	1
161M	27	S	1	161M	19	S	1
162A	31	S	1	162A	33	S	1
162M	20	S	1	162M	23	S	1
169A	36	S	1	169A	33	S	1
169M	32	S	1	169M	20	S	1
170A	25	S	1	170A	41	S	1
170B	38	S	1	170B	15	I	1
170M	28	S	1	170M	18	S	1
171A	36	S	1	171A	34	S	1
171M	22	S	1	171M	25	S	1
172A	23	S	1	172A	32	S	1
172B	27	S	1	172B	25	S	1
172C	33	S	1	172C	22	S	1
172M	34	S	1	172M	18	S	1
173A	18	I	1	173A	30	S	1
173B	36	S	1	173B	21	S	1
173M	8	R	1	173M	23	S	1
176A	35	S	1	176A	29	S	1
176M	33	S	1	176M	30	S	1
180A	11	R	1	180A	37	S	1
180M	48	S	1	180M	27	S	1
181A	34	S	1	181A	30	S	1
181M	31	S	1	181M	21	S	1
182A	26	S	1	182A	15	I	1
182B	0	R	1	182B	32	S	1
182M	20	S	1	182M	35	S	1
183A	31	S	1	183A	27	S	1
183M	25	S	1	183M	24	S	1
184A	32	S	1	184A	30	S	1
184M	17	I	1	184M	24	S	1

185A	16	I		1	185A	27	S	1
185B	31	S		1	185B	27	S	1
185C	17	I		1	185C	28	S	1
185M	13	R		1	185M	24	S	1
186A	35	S		1	186A	31	S	1
186M	13	R		1	147A	36	S	0
147A	49	S		0	147B	44	S	0
147B	41	S		0	147M	39	S	0
147M	37	S		0	148A	43	S	0
148A	36	S		0	148B	39	S	0
148B	35	S		0	148M	38	S	0
148M	39	S		0	159A	33	S	0
159A	22	S		0	159M	27	S	0
159M	34	S		0	160A	39	S	0
160A	25	S		0	160M	23	S	0
160M	24	S		0	164A	34	S	0
164A	27	S		0	164B	24	S	0
164B	37	S		0	164M	23	S	0
164M	41	S		0	174A	37	S	0
174A	25	S		0	174B	25	S	0
174B	33	S		0	174C	34	S	0
174C	34	S		0	174M	31	S	0
174M	31	S		0	175A	30	S	0
175A	12	R		0	175M	22	S	0
175M	35	S		0	177A	28	S	0
177A	0	R		0	177M	27	S	0
177M	13	R		0	178A	38	S	0
178A	23	S		0	178M	29	S	0
178M	43	S		0	179A	30	S	0
179A	38	S		0	179B	35	S	0
179B	39	S		0	179C	39	S	0
179C	25	S		0	179M	18	S	0
179M	13	R		0				

Appendix V: Rifampin and Doxycycline ZOI organized by category

Gram Positive Coccus	Gram Positive Bacillus	Gram Negative Bacillus	Total
253	104	20	377
67.1%	27.6%	5.3%	100%

Appendix W: Comprehensive Gram Staining Results

Student Participation Questionnaire and Authorization for Participation

1. Have you been prescribed antibiotics in the last two years?   _____YES _____ NO

2. If yes, can you recall which antibiotic and the duration?

   Have you **ever** taken antibiotics specifically for acne? ____YES _____NO

3. During the course of antibiotic treatment, did you also use a topical face wash or gel? Did your dermatologist prescribe this treatment? _____ YES _____NO

4. Can you list the active ingredient found in your daily face wash?

5. Has your acne improved? _____ YES _____ NO

6. Which therapy or face wash has worked the best?

7. Briefly describe your skin. Do you break out often? Have you ever had acne?

In today's world, antibiotics are becoming more and more prevalent. This prevalence however, also leads to a development in antibiotic resistance. Persons battling acne are prescribed antibiotic regimens that may last for two months all the way up to a year. Recent studies suggest that antibiotic resistance can develop in as little as 12 weeks. Most acne patients are not rid of the condition prior to stopping antibiotic use, leaving colonies present that have now developed resistance to that specific antibiotic. Increased research on this subject may pave the way for improvement in treating acne. We will be collecting a sample of your skin cells using a non-evasive swabbing protocol. Three samples will be obtained with a cotton swab and tested for antibiotic resistance development as well as a survey of the bacteria present. This experiment will be conducted at Pittsburg State University and will not include any of your personal information, other than your signature for consent.. This research is completely voluntary and you may withdraw at any moment, but if you are giving consent to participate, please sign and date below.

Signature _____   Date_____

Appendix X: Informed Consent. Each student completed and signed this form.