Genetic Homology Among Local Methicillin-Resistant *Staphylococcus aureus* Strains

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ABSTRACT

Each year in the United States at least 2 million people become infected with bacteria that are resistant to antibiotics; at least 23,000 people die each year as a direct result of these infections. It will always be priority to develop new antibiotics and diagnostic tests. The rationale of this senior thesis was to identify major genomic difference between low and high methicillin-resistant *Staphylococcus aureus* strains. EcoR1 and HindIII were used in restriction digestion to identify genome difference between each strain. Neither EcoR1, HindIII, nor EcoR1 + HindIII showed any major genomic differences between low and high resistant strains. Further studies should be conducted to quantitate gene expression between resistant levels.

INTRODUCTION

One of the greatest advancements in therapeutic medicine of all time was the introduction of penicillin in the 1940's. A "miracle" drug that could defeat infections humans once succumbed to. Although, Sir Alexander Fleming saw the fallout himself, as he stated this at the end of his Nobel Prize speech, "There is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."[11] The issues associated with antibiotic resistance have inspired many agency and governmental reports since 1998, along with multiple sets of usage guidelines.[6] Each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics. At least 23,000 people die each year as a direct result of these infections.[19] In an Active Bacterial Core surveillance of methicillin-resistant Staphylococcus aureus (MRSA), by the Center for Disease control, 62,500 cases were documented in 2011. An estimated 11,285 people died from the infection.[2] The World Health Organization reported in 2007, nine countries in Central and South America where 26-50% of *Staphylococcus aureus* infections were resistant and six countries where >51% of isolates were resistant.[15] Antibiotic resistance also adds considerable and avoidable costs to the U.S. healthcare system. Estimates vary for the total economic cost of antibiotic resistance to the U.S. economy, but many have ranged as high as $20 billion in direct healthcare costs.[19] It is important to continue to develop new, efficient antibiotics, but to also investigate genomic trends of resistant bacteria.

The use of antibiotics is the single most important factor leading to antibiotic resistance. Over 50% of all antibiotics prescribed are not needed. Another factor leading to the growth of antibiotic resistance is the spread of resistant strains of bacteria from

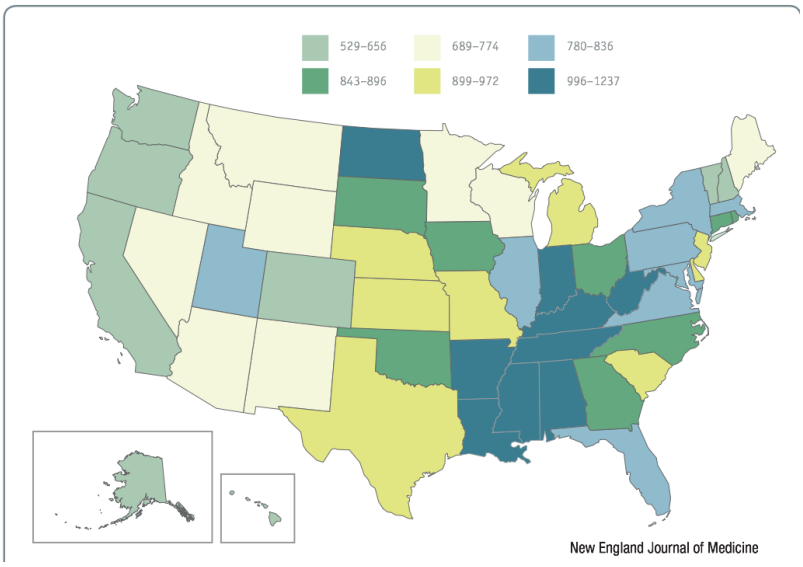
person to person. The Center for Disease Control (CDC) uses four core actions that will help fight these deadly infections: preventing infections and preventing the spread of resistance, tracking resistant bacteria, improving the use of antibiotics, and promoting the development of new antibiotics and diagnostic tests for resistant bacteria.[19]

To prevent infections, prevention must start in healthcare facilities. Almost all Americans will receive care in a medical setting at some point, so the problem can affect anyone. It compounds the problem that those in the healthcare setting most likely have weakened immune systems. This makes them more vulnerable to contracting an antibiotic resistant infection. The CDC works to prevent antibiotic resistance in health care setting by providing a tracking system and antibiotic prescribing patterns at national, regional, and local levels. These programs can help create guidelines to prevent infections and spread of resistance.[19]

The data gathered by the CDC can help track resistance patterns. For example, if there are particular risk factors that cause some people to become more susceptible than others. The CDC uses three main programs within the emerging infections program that are tracking networks: Active Bacterial Core Surveillance, Healthcare-Associated Infections-Community Interface and Foodborne Diseases Active Surveillance Network.[19]

To decrease antibiotic resistance the primary factor must be addressed, improper use of antibiotics. When antibiotics are prescribed improperly it doesn't only fail the patient but might cause harm. Antibiotics, like any drugs, have side effects that can interfere and interact with normal body functions and create habitats for more serious bacteria to grow. Antibiotic trends vary greatly from state to state, a reason for the surveillance from the CDC (Figure1). It is important all antibiotics have a dose, duration,

and indications. Also, the evaluation of cultures as soon as possible to assess antibiotic selection and a review period 48-72 hours to assess the antibiotics performance. A University of Maryland study showed $17 million dollars could be saved over eight years if "antibiotic stewardship" programs were implemented.[19]



http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf#

Figure 1: The frequency with which doctors prescribe antibiotics varies greatly state to state.

As programs are set up to decrease antibiotic resistance, it is important the development of new antibiotics and diagnostic tests continue. Antibiotic resistant is a natural evolutionary process; therefore, it will never be stopped. It is important that steps are taken to slow the process. New antibiotics will always need developed to continue with the trends of resistant bacteria. . Unfortunately antibiotic formation has been on the decline since the 1980's (Figure 2).[19] Governmental agencies have increased standards and regulations an antibiotic must meet to be approved. Also, pharmaceutical companies are pursuing long-term medications, instead of short-term medications like antibiotics. It

is more profitable to study and produce medications that are used for a lifetime or longer periods (i.e. blood pressure and cholesterol). Antibiotics are usually prescribed for 7 to 14 days.

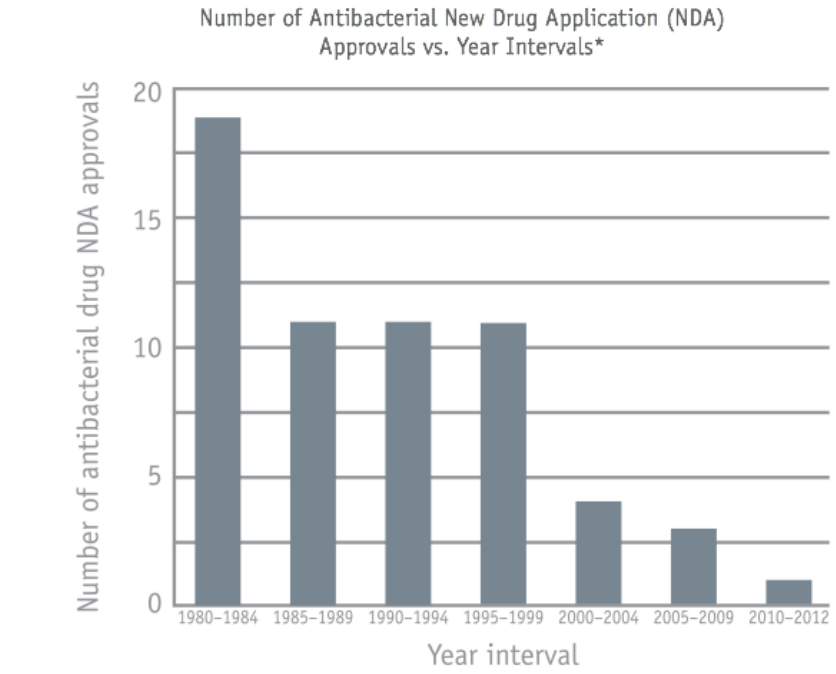


Figure 2: The number of new antibiotics developed and approved has steadily declined.

Antimicrobial drugs can be categorized based upon their mechanisms of action. A drug can inhibit cell wall synthesis. The drugs in this category are selectively toxic to certain fungal and bacteria, organisms that have cell walls. By preventing the cross-linkage of N-acetylmuramic acid (NAM) subunits, the cell wall's major structural component, peptidoglycan is formed inaccurately. This creates a weakened cell wall that lyses due to ineffective osmotic regulation. The most common drugs among these are β-lactams, such as penicillins and cephalosporins. Methicillin is an alteration of penicillin

that allows it to remain stable in the low pH of the stomach, to absorb more efficiently in the intestinal tract and is less resistant to deactivation by bacterial enzymes. Vancomycin interferes with alanine-alanine bridges that link the NAM subunits in many gram-positive bacteria. Bacitracin blocks the transport of N-acetylglucosamine (NAG) and NAM from the cytoplasm (Table 1).[1]

Other types of drugs inhibit protein synthesis to affect a pathogen. Proteins are a vital part of cell structure and regulation. Prokaryotic ribosomes differ from eukaryotic ribosomes by size: prokaryotic ribosomes are 70S and composed of 30S and 50S subunits; whereas, eukaryotic ribosomes are 80S with 60S and 40S subunits. This allows antimicrobial agents to selectively target bacterial protein translation. Linocosamides and macrolides bind to a portion of 50S subunit, preventing movement of the ribosome from one codon to the next. Many other antibiotic classes, such as aminoglycosides and tetracyclines work to inhibit protein synthesis, also.[1]

Antimicrobial agents can also work to disrupt cytoplasmic membranes. Most classes in this category incorporate themselves in the membrane and damage its integrity. Gramicidin forms pores in the membrane and allows cations to cross freely. Amphotericin B attaches to ergosterol, a lipid part of fungal membranes, disrupting the membrane causing lysis of the cell. Polymyxin is able to destroy the cytoplasmic membranes after entering into the cell.[1]

Another mechanism used is the inhibition of metabolic pathways. Metabolic pathways can be defined as the sum of all chemical reaction that takes place in an organism. Anti-metabolic agents are able to interfere with electron transport. Trimethoprim blocks the metabolic pathway in the formation of folic acid. Sulfonamides

bind irreversibly to the enzyme that produces folic acid. Folic acid is needed in prokaryotes for the formation of nucleic acids and other important functions.[1]

The last mechanism of action an agent can be categorized in is the inhibition of nucleic acid synthesis. This group is considerably smaller than the previous stated since prokaryotes and eukaryotes share very similar constructed DNA. Nucleotide analogs incorporate into the DNA or RNA and distort the shape of the nucleic acid molecules. This prevents further replication, transcription, or translation. Rifampin binds to bacterial RNA polymerase, preventing transcription of RNA. Fluoroquinolones are unique because they act against prokaryotic DNA specifically. Each agent inhibits DNA gyrase, the enzyme necessary for correct coiling and uncoiling of replicating bacterial DNA.[1] DNA gyrase is found in prokaryotes and some eukaryotes. The enzymes are not entirely similar in structure or sequence, and have different affinities for different molecules. Other topoisomerases are responsible to prevent supercoiling in humans.

Mechanism of Action	Drug Example
Alteration of cytoplasmic membranes	gramicidin, amphotericin B, polymyxin
Inhibition of metabolic pathways	sulfonamides, trimethoprim
Inhibition of nucleic acid synthesis	rifampin, fluoroquinolones
Inhibit protein synthesis	macrolides (erythromycin, azithromycin), lincosamides (clindamycin), tetracyclines, aminoglycosides
Inhibition of cell wall synthesis	β-lactams (penicillins,cephalosporins) vancomycin, bacitracin

Table 1: Antibacterial drugs and mechanism of action.

The improper use of antibiotic agents has driven rapidly evolving infectious organisms to combat the effectiveness of the drugs designed to stop or slow their growth. Most bacteria gain resistance through mutations of chromosomal genes or by gaining resistant genes carried on R-plasmids by transformation, transduction, or conjugation.

A plasmid is a small molecule of DNA that replicate independently of chromosomes in prokaryotic cells. Plasmids are circular and 1-5% the size of a prokaryotic chromosome. Often they have a few thousands base pairs but can be up to a few million base pairs. Each plasmid carries specific information for its own replication and genes that are not essential for normal metabolic growth of a cell. Plasmid maps are

used as a graphical depiction of where those genes are located (Figure 3). Many types of plasmids exist: Fertility (F) factors, Resistance (R) Factors, Bacteriocin factors, and Virulence plasmids.[1]

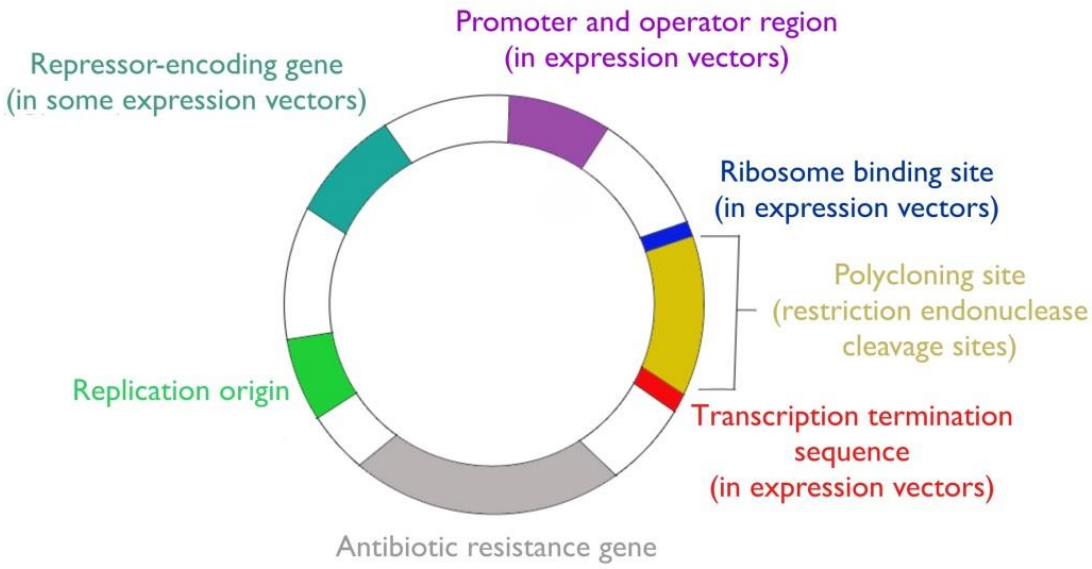


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Figure 3: Basic plasmid map construction

Fertility factors carry genes for conjugation, a process that transfers genes from one bacterial cell to another. Resistance factors carry genes for resistance to one or more antimicrobial drugs. These cells can transfer resistance factors to other cells, which then acquire resistance to the same antimicrobial agent. These transfer mechanisms are called horizontal gene transfer and a few methods exist: transformation, transduction, and conjugation.[1]

In transformation, a recipient cell takes up DNA from the environment. Frederick Griffith discovered this process in 1928 while trying to develop a vaccine for *S. pneumoniae*. He was working with two separate strains of *Streptococcus pneumoniae* and

the first strain (S strain) had a protective capsule that allowed them to bypass the body's defense system and the second (R strain) didn't have a capsule. The S strain caused immediate death in a mouse, bypassing its immune system. The R strain didn't cause any harm to the mouse and was destroyed by the immune system. He heat-treated the S strain and injected the mouse again and it wasn't harmed. He then injected the heat-treated S strain + the live R strain and the mouse died. Some of the living R strains took up the DNA from the S strain and incorporated it into their genome. This turned the once R strains into S strains, producing capsules, and killing the mouse. In 1944, Avery, McLeod, and McCarty extracted chemicals from the S strain and determined the transforming agent was DNA. *Staphylococcus* strains are one of the few that have the ability to take up DNA from their environment naturally.[1]

The second method of horizontal gene transfer is transduction. It involves the transfer of DNA from one cell to another via a replicating virus. To replicate, a bacteriophage (a virus that infects bacteria) attaches to a bacterial host cell and injects its genome into the cell. Enzymes within the bacteriophage degrade the cell's DNA, so the phage genome controls the cell's functions. It synthesizes new phage DNA and phage proteins. Eventually the cell lyses, releasing daughter and transducing phages. Transducing phages are phages that have mistakenly incorporated the host DNA into the phage DNA. Transduction occurs when the transducing phage injects its DNA into a new host cell. That host cell incorporates the DNA into its chromosome recombination (Figure 4).[1]

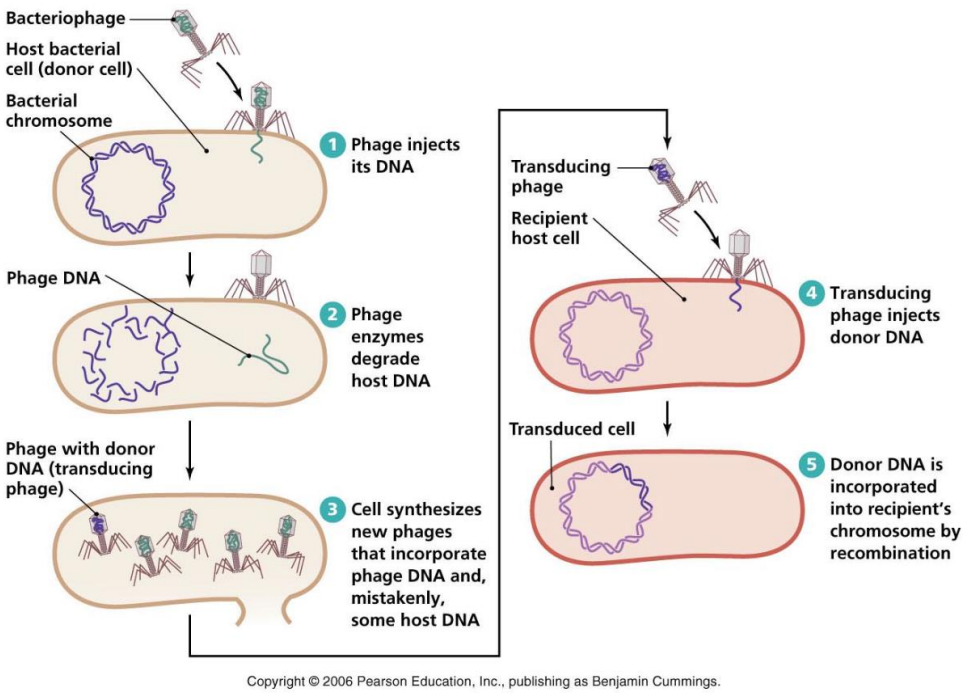


Figure 4: Transduction method of horizontal gene transfer.

The final horizontal gene transfer is conjugation. Conjugation requires physical contact between the donor and recipient cell. It is mediated by a conjugation pilus; a rodlike structure that extends from the surface of a cell. The gene encoding for conjugation pili is located on the F plasmid. Those with an F plasmid are called F+ cells. Conjugation begins when the sex pilus connects a F+ cell to a F- cell and draws the cells together. Once they make physical contact, one strand of the F plasmid transfers to the recipient (F-) and it synthesizes a complementary strand of F plasmid DNA (becoming a F+ cell) (Figure 5). Conjugation occurs in a number of species of bacteria, meaning it occurs among multiple different strains.[1]

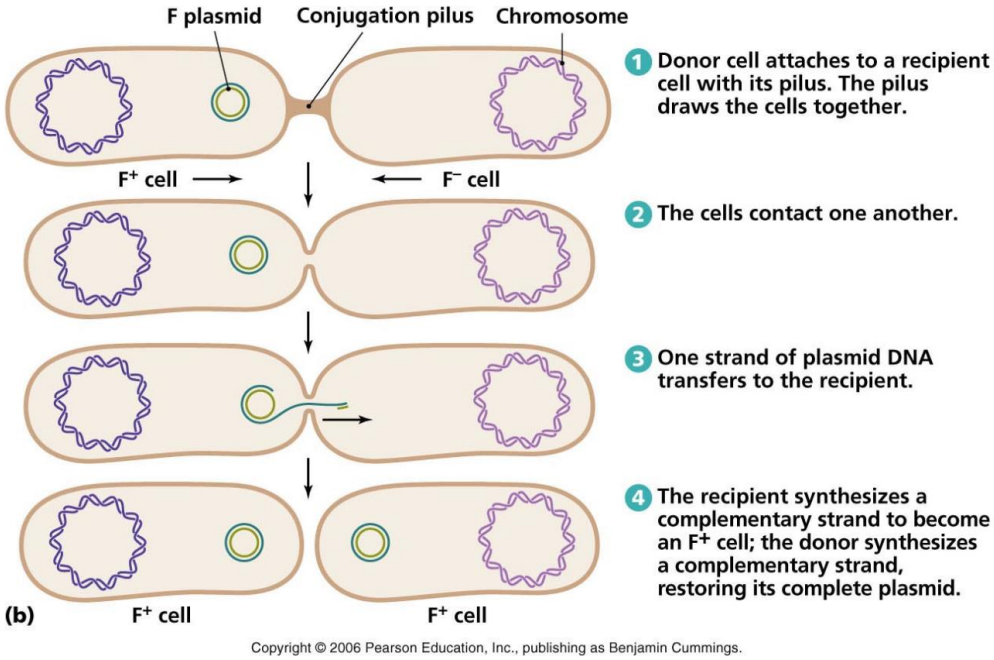


Figure 5: Conjugation method of horizontal gene transfer.

A typical antimicrobial must cross the cell's wall, cross the cytoplasmic membrane, and bind to its target. There it is able to inhibit the bacteria. At least five methods of resistance are acknowledged today: resistance cells produce an enzyme that destroys or deactivates a drug, resistant cells slow or prevent the entry of the drug into the cell, resistant cells alter the target of the drug so it cannot attach to it, resistant cells can alter their metabolic chemistry, resistant cells can pump the agent out of itself or a bacterium.[1]

Resistant cells may produce an enzyme that destroys or deactivates a drug. A common example is β-lactamases, which are enzymes that break the β-lactam rings of penicillins, cephalosporins, and similar molecules. Without the β-lactam ring these drugs are inactive. Over 200 different lactamases have been identified. Their genes are carried on R-plasmids.[1]

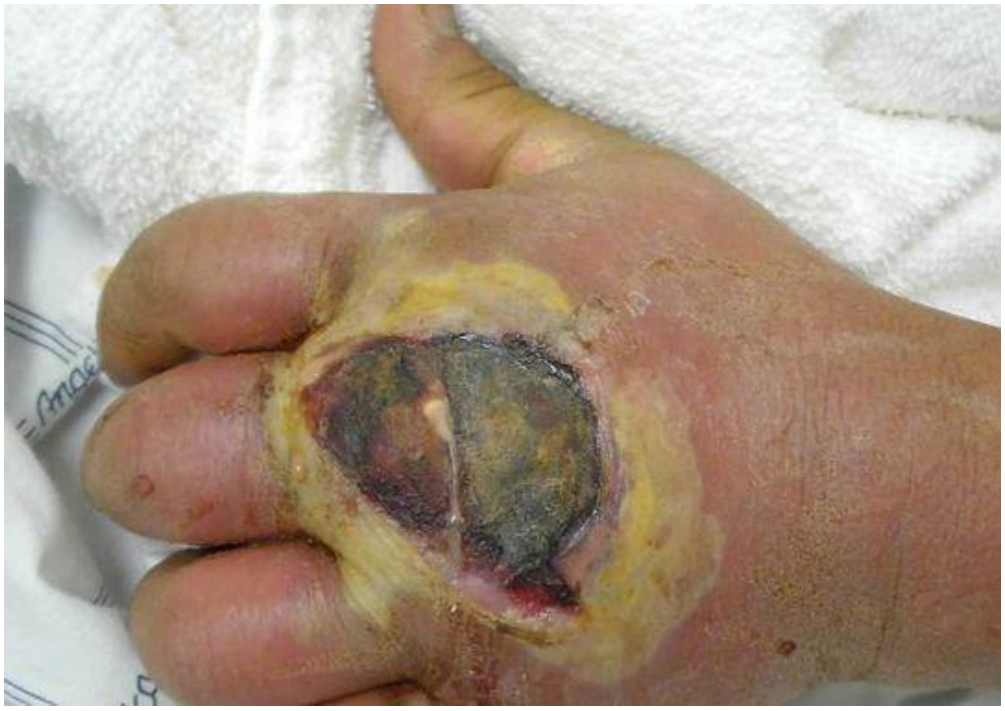
Resistant cells can slow or prevent the entry of the drug into the cell. This mechanism involves changing the structure or electrical charge of the cytoplasmic membrane proteins. These proteins control channels and pores that allow access into the cell. Resistance against tetracycline and penicillin occurs via this mechanism.[1]

Resistant cells may alter their metabolic chemistry. It can become resistant to a drug by producing more enzyme molecules for the affected metabolic pathway, reducing the drug's effectiveness. Cells become resistant to sulfonamides by stopping the synthesis of folic acid and absorbing it from the environment instead. Folic acid is important to synthesize nucleic acids and carry out important functions within the cell.[1]

Resistant cells may pump the drug out of the cell before it can act. These pumps are called efflux pumps, which are powered by ATP and can pump more than one type of drug from a cell. Some bacteria can become multi-drug resistance by utilizing many efflux pumps to pump out more than one drug.[1]

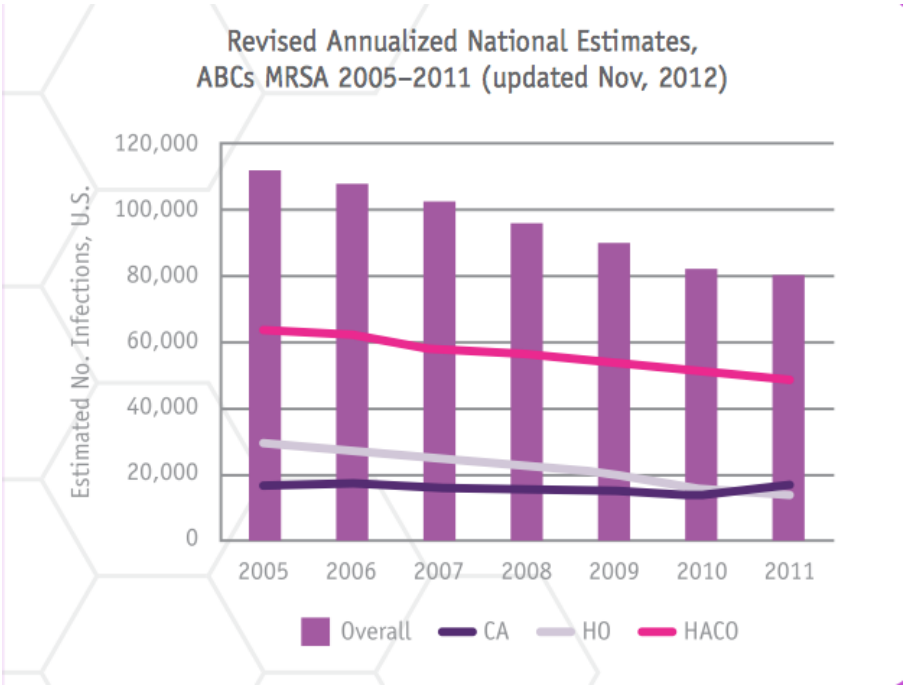
Staphylococcus aureus is a gram-positive coccus. It is one of over 30 different strains of Staph, some of which are normally found on our skin. *Staphylococcus aureus* is the leading cause of skin and soft tissue infections, causing cellulitis and abscesses (Photo 1).[8] Methicillin-resistant *Staphylococcus aureus* gets its name because it no longer is inhibited by the antibiotic methicillin. Most MRSA strains are multi-drug resistant, being resistant to multiple classes of antibiotics. It once was primarily hospital-associated (HACO), but in recent years an increased number of cases have become community-associated infections (CA) (Figure 6).[9] Levels of resistance are determined by minimum inhibitory concentrations (MIC). MRSA strains can be categorized as low resistance, medium resistance, or high resistance. If the MIC is < 1 it is low resistance, 1-2 is

medium resistance, and >2 is high resistance. MRSA gains its immunity by the formation of β-lactamases, breaking down the microbial used to inhibit its growth. The most common antibiotic to combat the infection now is Vancomycin, inhibiting cell wall synthesis but by a different mechanism than the penicillins. Vancomycin-resistant *Staphylococcus* infections are becoming more common and have a very high virulence. Three new antibiotics have been approved by the FDA to combat VRSA: Zyvox, Cubicin, and Synercid.[3] Those are the only three remaining antibiotics able to combat the infection.



http://www.usatoday.com/story/news/nation/2013/12/16/mrsa-infection-community-schools-victims-doctors/3991833/

Photo 1: A cutaneous abscess caused by MRSA on a hand.



http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf#

Figure 6: Severe MRSA infections mostly occur during or soon after inpatient medical care.

Methicillin resistance is the most common resistance in *Staphylococcus aureus*. It is often carried on the plasmid, pUB101. The plasmid is 21,845bp and blaZ, blaR1, and blaL genes are located on this plasmid.[16] The methicillin resistance is carried by the gene blaZ; this gene encodes β-lactamase, which deactivates the β-lactam ring of methicillin. This enzyme is synthesized inside the cell and then secreted outside the cell when exposed to β-lactam antibiotics. Two other complement enzymes, an anti-repressor blaR1 and repressor blaL control blaZ. β-lactamase synthesis requires sequential cleavage of the regulatory proteins, blaR1 and blaL. It is hypothesized that the cleaved protein of BlaR1 functions as a protease, cleaving the repressor blaL. This allows blaZ to synthesize the enzyme β-lactamase.[5] The gene blaZ is found at 16071bp to 16916bp. The complement gene blaL is found between 13859bp and 14239bp. BlaR1 is found between 14229bp and 15935bp[16].

Another resistance that is often carried among methicillin resistance is macrolide resistance. One specific macrolide often used to treat pediatric MRSA is clindamycin. Many MRSA's carry a clindamycin resistance. It is carried on the plasmid, pI5S5. The plasmid is 37,285bp and carries multiple erm genes, specifically ermC.[17] ErmC encodes for methylase. Methylase is an enzyme that adds methyl groups to nucleic acids. Methylase creates a structural change to the rRNA, preventing macrolides from binding. This allows the synthesis of bacterial proteins to continue.[7] The gene is found between 1858bp and 8592bp.[17]

The purpose of the research was to identify major genomic differences between low and high resistant (Table 2) MRSA by analyzing DNA fragments on a gel electrophoresis after a restriction digestion was performed. It is hypothesized that major genomic difference will occur between the low and high strains. It is expected that mutations would have occurred between the two levels of resistant strains causing them to respond differently to concentrations of antibiotics.

Strain	Minimum Inhibitory Concentration (Oxacillin)	Interpretation
1	>2	High Resistance
2	>2	High Resistance
3	>2	High Resistance
4	>2	High Resistance
5	>2	High Resistance
6	>2	High Resistance
7	>2	High Resistance
8	0.5	Low Resistance
9	>2	High Resistance

Table 2: The strains used with MIC and resistance levels.

PROCEDURES

Part 1: Restriction digestion and gel electrophoresis

To determine if major genome differences exist between low, medium, and high strains of MRSA a restriction digestion was performed followed by gel electrophoresis.

Part 1A: Formation of pellets

1. Multiple MRSA strains were obtained from a local hospital and inoculated in tryptic soy broth (TSB) and incubated for 24 hours at 37^{o}C. The strains were swabbed onto tryptic soy agar slants to maintain the bacteria long-term.
2. Pellets were made from TSB by transferring the broth to 10mL centrifugation tubes and centrifuging at 5,000 rpm for 10 minutes. The excess broth was poured off into a beaker, then pipetted off to remove the final excess of broth.
3. The pellets were frozen at -20^{o}C for later use in DNA extraction. All materials that were contaminated were autoclaved at 15 psi for 15 minutes at 121^{o}C.

Part 1B: DNA Extraction – Gen Elute Five-Minute Plasmid Miniprep Kit (Appendix 1)

1. The lysis reagents were prepared by chilling the lysis reconstitution solution on ice for 5-10 minutes. The kit size was 50 preps, so 3mL of lysis reconstitution solution was added to the lysis reagent vial and mixed thoroughly until all the powder dissolved.
2. To lyse the culture 30uL of reconstituted and prechilled lysis reagent was added to 400µL of the bacteria culture in a 2mL collection tube. It was mixed briefly by inversion and incubated for 2 minutes at room temperature.

3. To prepare binding column a GenElute Miniprep Binding Column was added to the 2mL collection tube. 500µL of column preparation solution to each column and spun for 10 seconds.
4. To bind the DNA 400µL of binding solution was added to the lysate and inverted 15 times. 780µL of the mixture was transferred to a binding column seated in a 2mL collection tube and spun for 20 seconds.
5. To wash the column 700µL of wash solution was added to each column and spun for 20 seconds. Then, 200µL of wash solution was added to each column and spun for 30 seconds.
6. To elute the DNA the binding column was transferred to a clean 2mL collection tube and 40µL of elution solution was added directly to the surface of the filter and spun for 30 seconds to elute.
7. The DNA was stored in a -20^{o}C freezer for use in restriction digestion and gel electrophoresis.

Part 1C: Restriction digestion

1. Master mixes were prepared for each enzyme digestion, approximately 150µL A master mix was prepared for EcoR1, HindIII, and EcoR1 + HindIII. Each master mix was prepared by adding 10µL of the enzyme, 20µL of the provided buffer (EcoR1 buffer and 2.1 HindIII buffer), and 120µL of deionized water.
2. A labeling system was designed to distinguish each strain and restriction digestion performed. In 1.5mL centrifugation tubes, 15µL of each master mix and 5µL of each DNA sample, and 3µL of loading dye were added. Adding

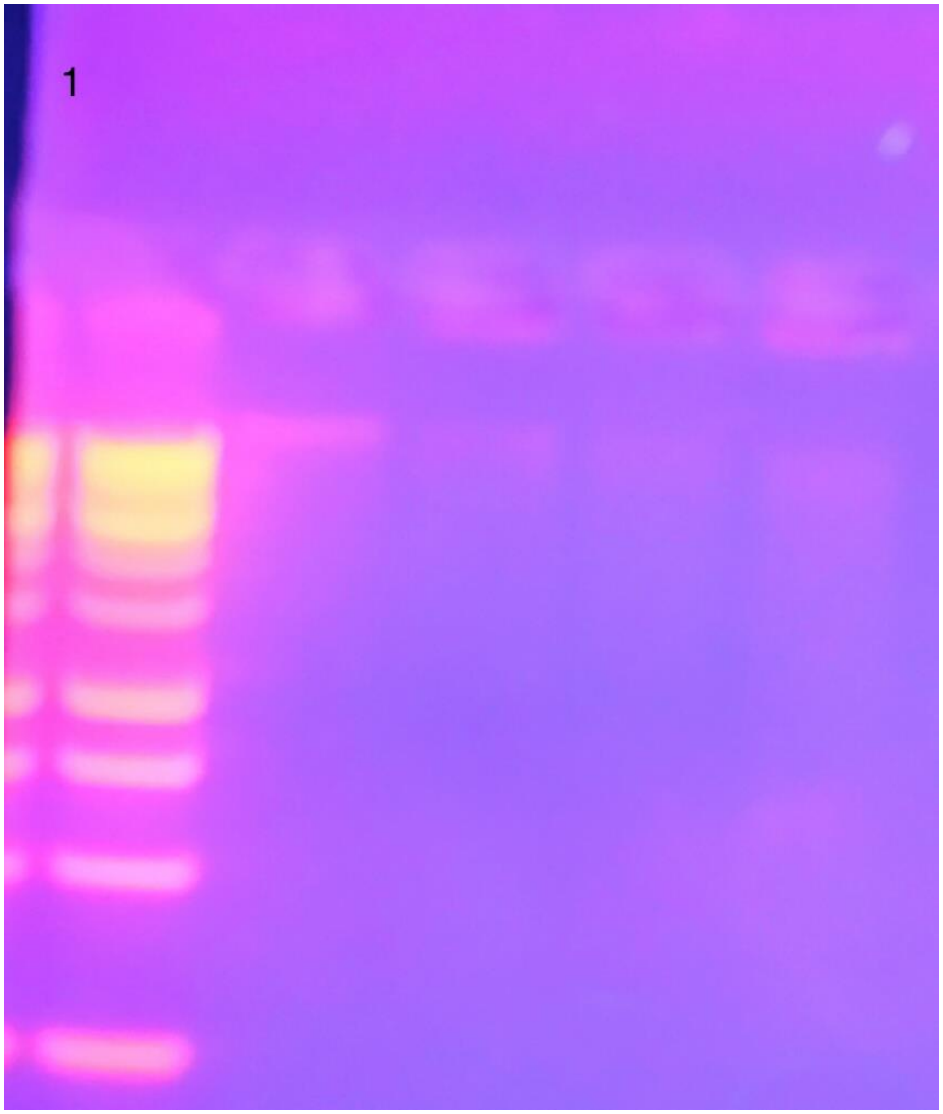
only the DNA created a control with buffer, loading dye, and DI water for the reaction.

3. The samples were vortexed and placed in a 37^{o}C water bath for two hours to activate enzymes. The samples were then moved to 67^{o}C water bath to discontinue the reaction for two hours.

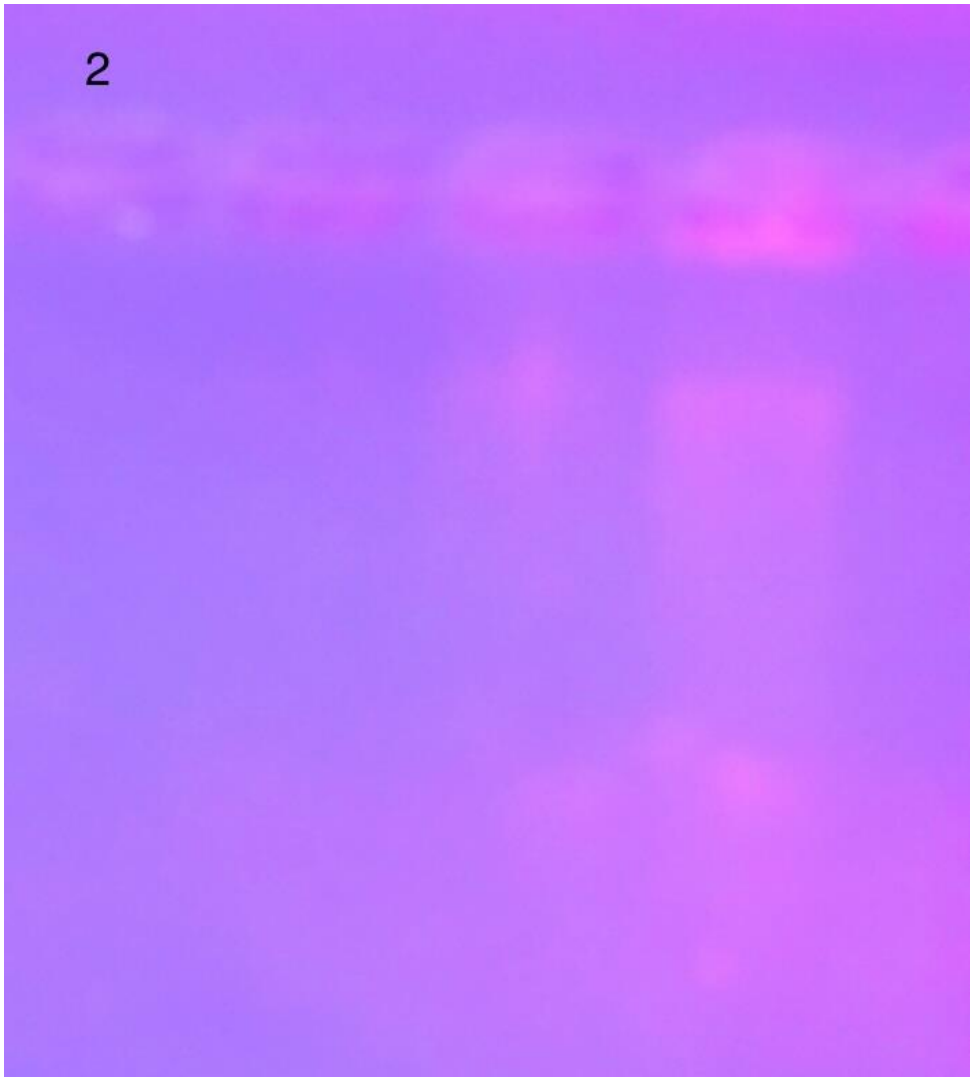
Part 1D: Gel Electrophoresis

1. Four 0.8% Agarose gels were mixed and poured into gel electrophoresis trays with a 12 well mold. The gels were aloud to solidify for four hours. A buffer was poured over the top of the gel to cover it completely.
2. 28µL of a ladder mix and 12µL of loading dye were used to create a ladder solution. The ladder was loaded (10µL) into the 1st well of each of the four gels.
3. Each restriction digestion (23µL) was transferred into individual wells and ran at 70 volts for two hours. Then, 110 volts for 50 minutes.
4. The gels were then placed on a UV light where results were recorded.

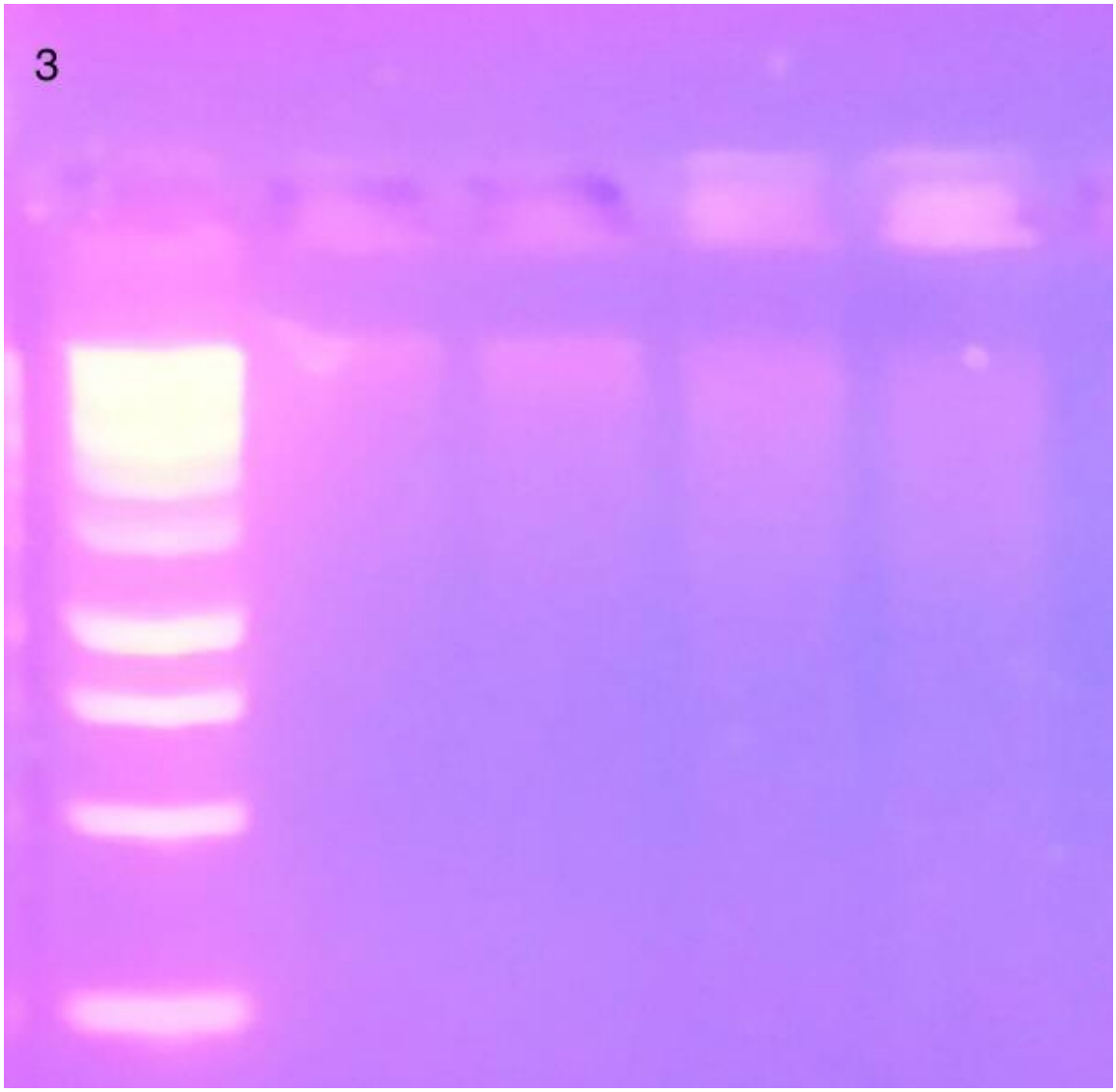
RESULTS



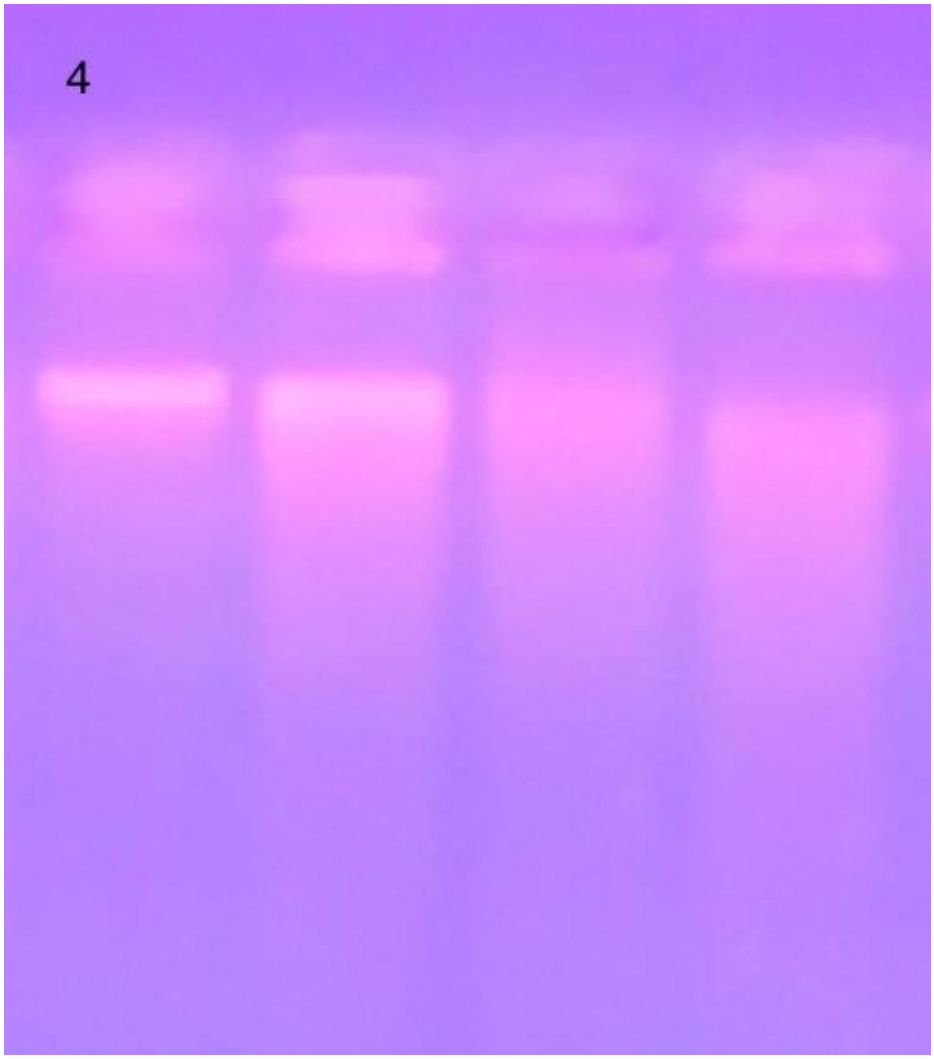
Strain 1 after restriction digestion. The first well was a ladder loaded at the beginning of each gel, the second well was the uncut control, the third well was EcoR1, the fourth well was HindIII, the fifth well was EcoR1 + HindIII.



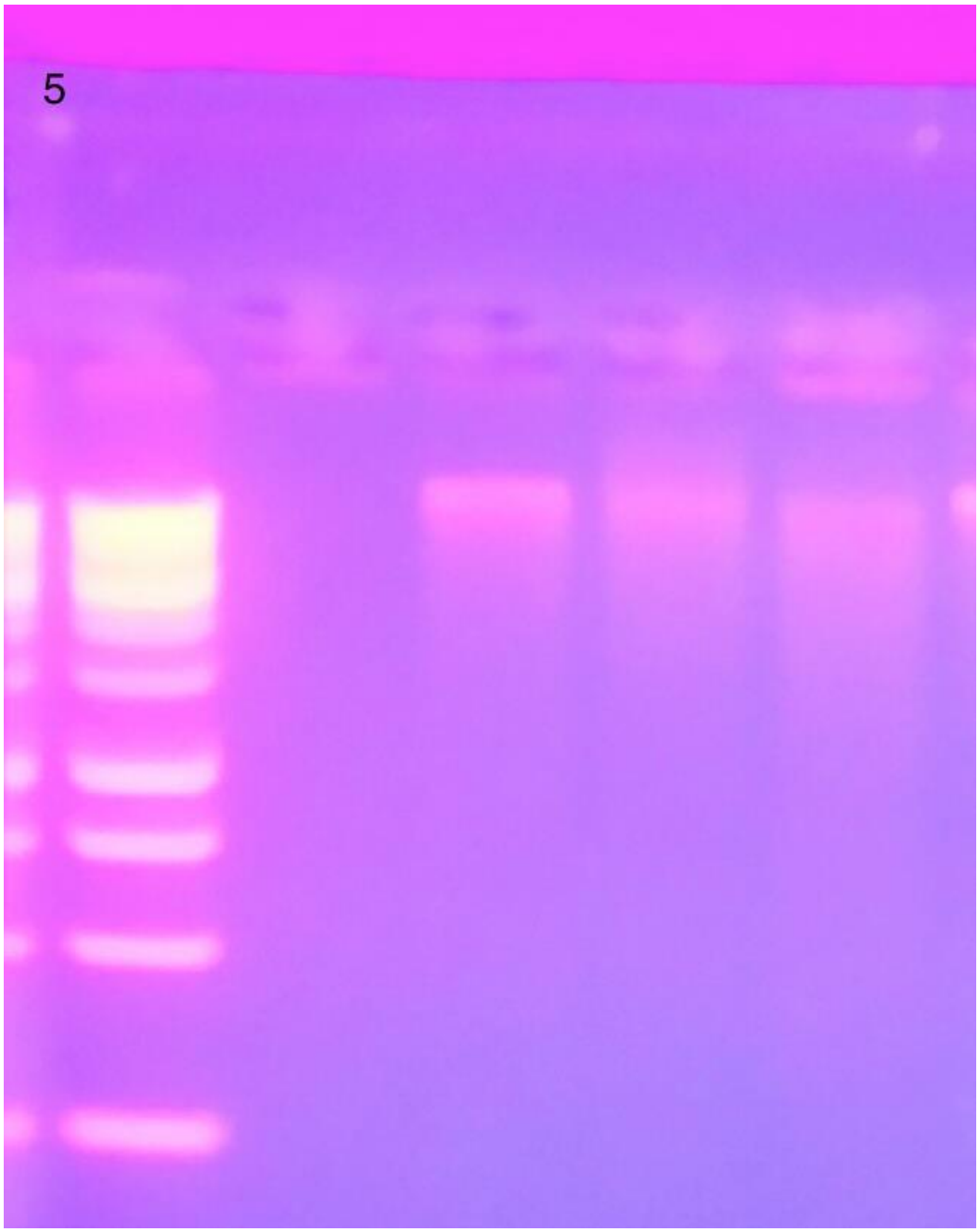
Strain 2 after restriction digestion. The first well is the uncut control, the second well is EcoR1, the third well is HindIII, and the fourth well is EcoR1 + HindIII.



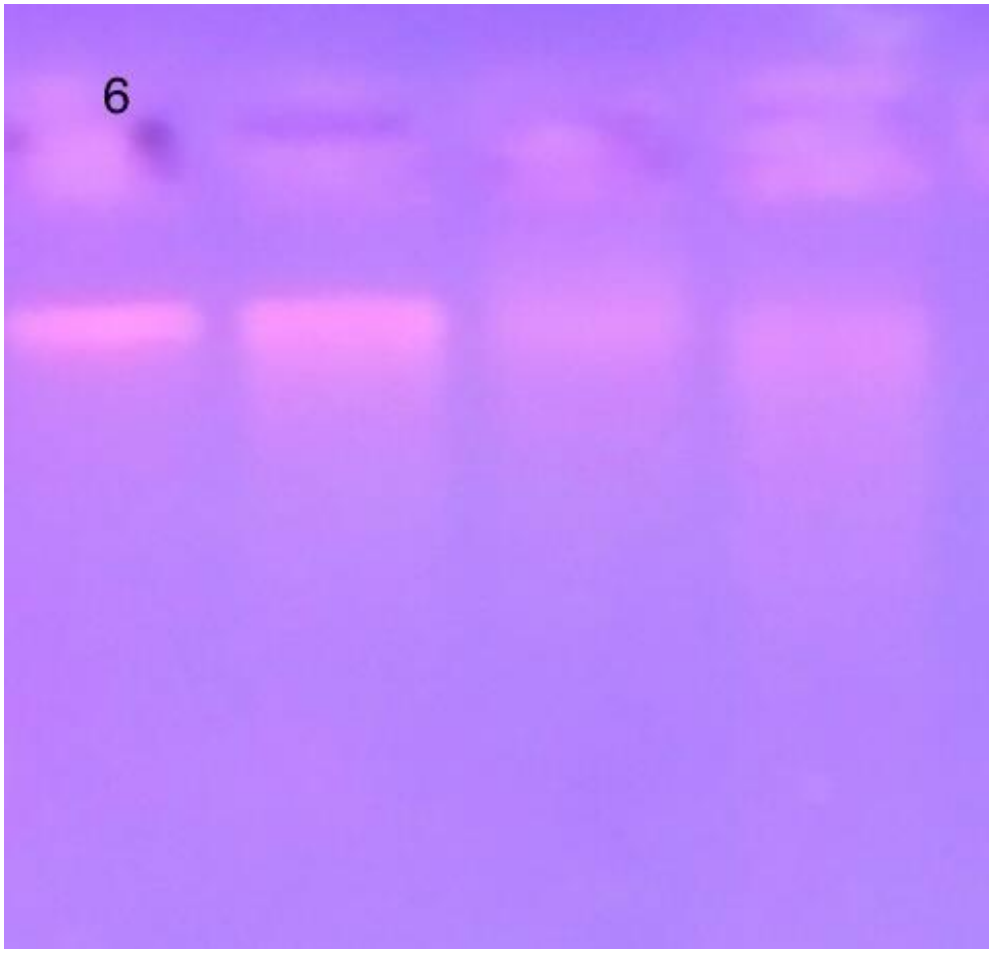
Strain 3 after restriction digestion. Wells 1-5 are as follows: ladder, uncut control, EcoR1, HindIII, and EcoR1 + HindIII.



Strain 4 after restriction digestion. Wells 1-4 are as follows: uncut control, EcoR1, HindIII, and EcoR1+ HindIII.

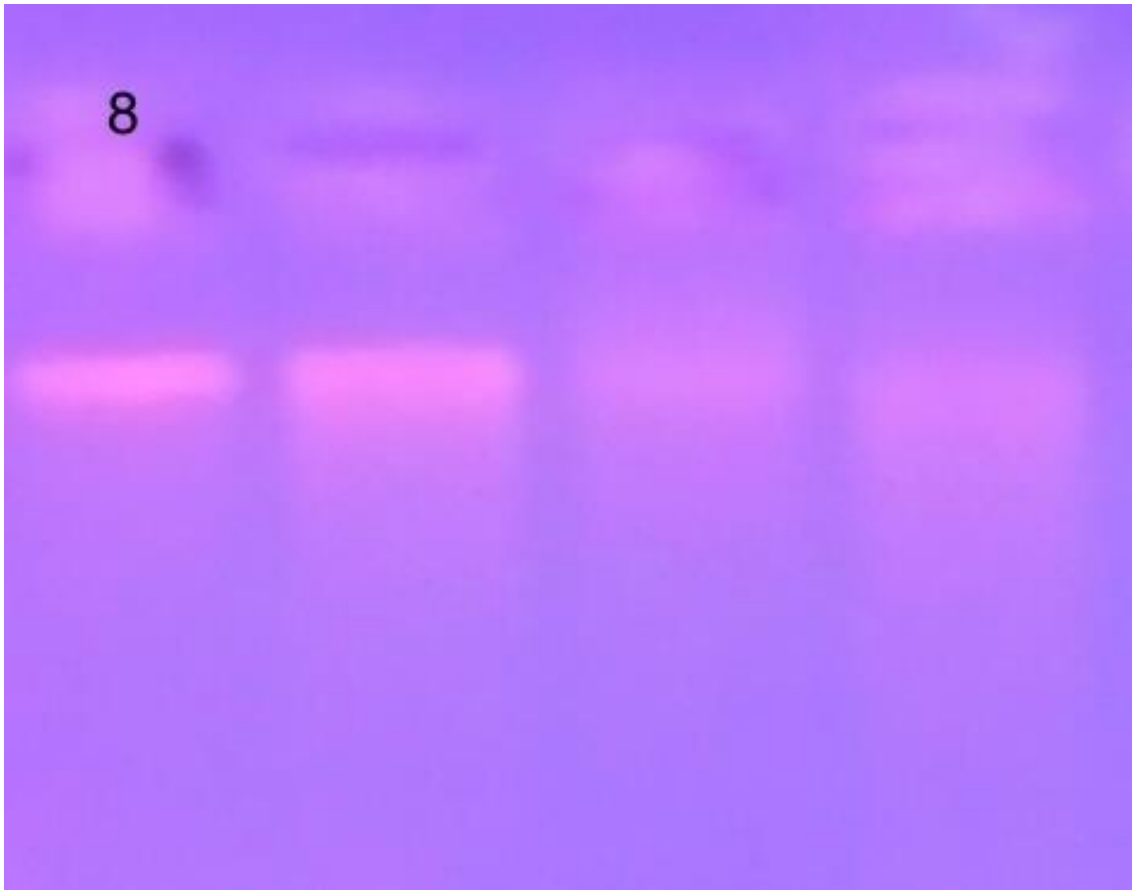


Strain 5 after restriction digestion. Wells 1-5 are as follows: ladder, uncut control, EcoR1, HindIII, and EcoR1 + HindIII.

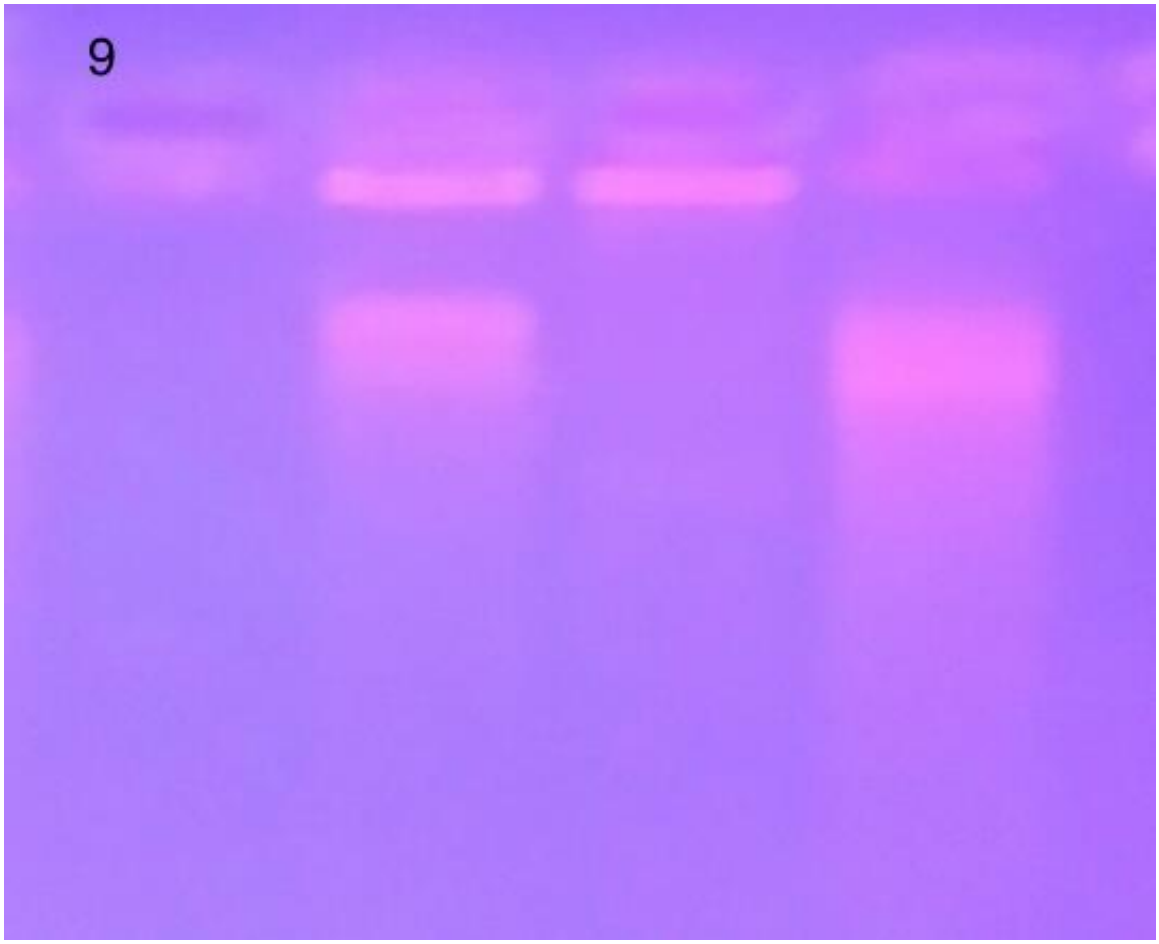


Strain 6 after restriction digestion. Wells 1-4 are as follows: uncut control, EcoR1, HindIII, and EcoR1 + HindIII.

Strain 7 after restriction digestions. Due to lack of well space strain 7 was split between two separate gels. Wells in 7.1 are the uncut control and EcoR1 left to right. In 7.2 they are HindIII and EcoR1 + HindIII left to right.



Strain 8 after restriction digestion. Wells 1-4 are as follows: uncut control, EcoR1, HindIII, and EcoR1 + HindIII.



Strain 9 after restriction digestion. Wells are as follows: uncut control, EcoR1, HindIII, and EcoR1 + HindIII.

DISCUSSIONS

The hypothesis that major genomic difference would occur between low and high strains was not supported. After restriction digestion, neither the high or low should unique cuts of the DNA. There was no plasmid difference between the two levels of resistance. In strain 2 after gel electrophoresis, only EcoR1 and HindIII DNA was present. In strain 5, the uncut control DNA wasn't present. In strain 9, the uncut control and HindIII DNA was not present.

If restriction digestion was performed again different restriction enzymes could be chosen. Restriction enzymes are specific to certain nucleotide sequences of DNA; therefore, if another restriction enzyme was chosen that targeted other sequences of nucleotides it is possible that the DNA could be cut. EcoR1 and HindIII were chosen because of their inexpensiveness and availability.

It would also be important to extract more DNA from each bacterial strain and add more into the restriction digestion. In multiple gels, DNA was not present. On the gels with DNA, ethidium bromide was used to try to intensify the fluorescence of the DNA under the UV light.

FUTURE STUDIES

Since major genomic differences were ruled out in the study, gene expression should be investigated between low, medium, and high resistant MRSA strains. Gene expression has an influential part in the response to environment in bacteria; therefore, this could be an attributing factor to the difference in response between resistant levels. The expression level should be determined by real-time polymerase chain reaction. First

fresh pellets would need to be made since RNA is very unstable. After pellets are made, procedures should be conducted to extra RNA according to Triton X procedures published by K. Sung et al.[18] Once the RNA is extracted it should immediately converted to cDNA by a kit that uses reverse transcriptase. Once the cDNA is collected it should be ran through real-time polymerase chain reaction. To create the primers for real time polymerase chain reaction primers should be made according to the gene sequence found on the plasmid. Materials and methods had been purchased and created to conduct the above research, but due to time restraints it was unable to be completed.

Another attributing factor to resistance that needs investigated is single nucleotide polymorphisms (SNPs) and promoter regions. Single nucleotide polymorphisms are unique genetic variations between separate strains. SNPs occur in DNA between genes and act as biological markers. These can contribute to the expression or inhibition of genes.[14] The promoter region is a specific region upstream from a gene that acts as a binding site for proteins. Promoter regions are responsible for turning a gene on or off. It is important to investigate the promoter regions of the bacteria to determine if the promoters have mutated, affecting gene expression.[12]

Another possible study would be intended to identify a new gene or acquired plasmid in the different levels of bacteria. A microarray would be loaded with the cDNA from each level of resistant bacteria to recognize if the same genes were being expressed and at what level the expression occurred. This would be another indication as to why bacteria acquire different levels of resistance and what mutation occurs to allow such evolution to happen.

INTROSPECTION

Albert Einstein once said, " It wouldn't be called research if we knew what we were doing…" I began research when I was 12 years old in the seventh grade and started competing in science research competitions during my eighth grade year. I continued research until my junior year in high school, forming relationships and research techniques that would aid in my success at Missouri Southern State University. Research teaches you life lessons, just like athletics or learning a new instrument or anything you put effort into. Research teaches you to be flexible, you design a plan and it never goes that way. People spend years researching a specific topic and it's usually at the very end they finally find something. In life you must be flexible, things will never go as planned. Research teaches you patience; it is a combination of months of work not a couple of days. By the time materials are bought, methods are designed, and you begin research it has been a time investment of months. Life will take patience, as I continue my education, career, and family. Research teaches you to be humble. As soon as you feel on top of the world because you've finally finished the procedures, it quickly pulls you down to Earth when no significant conclusions are found. Science is often a field where you're fishing for answers. It truly is by trial and error. It can build you up and break you down. All in all, research has continued to be a part of my life longer than I thought in the 7^{th} grade. I think it's certainly taught me lessons from day one that I started.

I had begun part 2 of the research (genetic expression), investing over 10 hours to complete the procedures up to the last test. Due Dr. Creamer away from the college and

time constraints when he returned we had to cut that portion of the research out of the paper. It is explained in the further research section.

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Procedure

***Note**: All spins at maximum speed (≥12,000 × g) and at room temperature; the suggested time in each step is only a minimum and can be extended for the sake of convenience.*

I. Spin Format

1. Lyse culture

Add **40 µL of reconstituted and prechilled Lysis Reagent (see Preparation Instructions)** to 400 µL of overnight culture in a 2-mL Collection Tube (provided). Mix briefly (3–5 seconds) by rapid inversion, vortexing, or pipetting up and down. Incubate at room temperature for 2 minutes. Culture will typically become clear after 2 minutes.

Note: The lysis incubation period may be reduced to 1 minute if maximum recovery is not required for the intended application. Dense cultures (OD_{600} >3.0) and some bacterial strains (such as XL1-Blue) may require longer incubation time for optimal plasmid recovery. In such cases, continue incubation until mixture clears. Longer incubation time does not have adverse effects on plasmid DNA quality.

2. Prepare Binding Column

Insert a GenElute Miniprep Binding Column into a 2-mL Collection Tube (provided). Add **500 µL of Column Preparation Solution** to each column and spin for 10 seconds. Decant the flow-through and insert the column back into the Collection Tube for subsequent use.

Note: This step can be carried out during the lysis incubation; however, it may be more convenient to prepare Binding Columns in batch earlier in the day prior to plasmid preparation if a large number of columns are needed. The pre-washed Binding Column will stay fresh for at least one day. This simple column preparation step ensures more uniform and higher plasmid yields.

3. Bind DNA

Add **400 µL of Binding Solution** to the lysate, cap the tube, and mix thoroughly by inverting at least 15 times. **Do not vortex.** Pour the mixture or pipette approximately 780 µL of the mixture to a pre-washed Binding Column seated in a 2-mL Collection Tube and spin for 20 seconds. Decant the flow-through.

Note: Inadequate mixing of the lysate mixture after adding Binding Solution might result in lower recovery. Plasmid DNA is stable in Binding Solution. When pouring the mixture into the column, there is no need to transfer every drop. Each column can only hold approximately 780 µL with the cap closed.

4. Wash column

Important Reminder: *Prior to first time use of the kit, be sure to add ethanol to the Wash Solution Concentrate (see Preparation Instructions).*

Add 700 µL of diluted Wash Solution to each column and spin for 20 seconds. Decant the flow-through. Add another **200 µL of diluted Wash Solution** to each column and spin for 30 seconds to wash and dry the filter. Carefully remove the column from centrifuge after the drying step to avoid splashing the flow-through liquid onto the dried column. If the flow-through liquid does contact the dried column, re-centrifuge the column for 20 seconds before proceeding to the elution step.

5. Elute DNA

Transfer the Binding Column to a clean 2-mL Collection Tube (provided). Add **40 µL of Elution Solution** (or water if desired) directly to the surface of the filter and spin for 30 seconds to elute. Plasmid DNA is now present in the eluate and ready for immediate use or storage at –20 °C.

Note: If a more concentrated plasmid DNA preparation is required, reduce the volume of Elution Solution to 30 µL. However, this may result in a reduction in the total plasmid yield.

1. Lyse w/ 40ml each culture
2. let sit 2min
3. Add 400ml Binding Sol. Invert
4. Add to column & spin 20sec
5. 700ml wash → 30sec spin
6. 200ml wash → 20 sec spin
7. 40ml Elute (In clean tube) sit 2min → spin 30 sec

APPENDIX 1

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