Dosage Based Antibiotic Cycling: Exploring a New Solution to Antimicrobial Resistance

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Abstract

Antimicrobial resistance is a well-documented public health crisis that continues to escalate and threatens our ability to successfully treat infections. Multiple solutions have been suggested, researched, and implemented to fight the development of antimicrobial resistance, but no current solution has proved adequate. Promising treatment methods against antimicrobial resistance are multidrug approaches promoting antibiotic heterogeneity. Dosage based antibiotic cycling is a novel treatment approach proposed in this study that combines the individual strengths of two existing multidrug approaches: traditional antibiotic cycling and combination therapy. This experiment utilizes a Kirby Bauer Disk Susceptibility test and a Colony Biofilm Assay to explore the effectiveness of dosage based antibiotic cycling as a solution against antimicrobial resistance by evaluating its ability to minimize the development of drug resistance in strains of *S. aureus* and *E. coli*. Antibiotic resistance results from dosage based antibiotic cycling will be compared to the results from corresponding monotherapy and combination therapy trials to determine if one strategy is more or less effective in preventing antimicrobial resistance than another. Results from this study, though inconclusive, open the door for the necessary further research.

Keywords: antimicrobial resistance, antibiotic heterogeneity, dosage based antibiotic cycling, Colony Biofilm Assay, Kirby Bauer Susceptibility test.

Introduction

Contextualizing Antimicrobial Resistance

Some form of antibiotic substances were utilized by the ancient civilizations, but it was not until the early-mid 1900's that widespread research, development, and use of antibiotics began in earnest (Aminov, 2010). This shepherded the beginning of the antimicrobial era in which common infections and minor injuries were no longer lethal threats to humans. Almost all individuals living in first world countries benefit from the protection afforded by an immense battery of antimicrobial drugs acting against the large array of fast-evolving pathogens (Garvey et al., 2016). Since their implementation, antibiotics have prevented the deaths of an innumerable amount of people across the globe. Despite this, continually emerging and escalating antimicrobial resistance (AMR) has the potential to undermine the perceived security of the antimicrobial era (Garvey et al., 2016). The O'Neill Report, a groundbreaking piece of research commissioned by the government of the United Kingdom and published in 2016, estimates that deaths caused by AMR could rise from approximately 700,000 deaths per year to close to 10 million deaths per year by 2050 (Garvey et al., 2016; Baker et al., 2018). The World Health Organization has warned that without urgent action against the threat of AMR, we are heading for a post-antibiotic era where common infections and minor injuries are once again lethal (*Antibiotic resistance*, 2020).

Antimicrobial resistance is a well-documented, increasing threat to global public health. Resistance evolves in pathogens via spontaneous mutation or horizontal gene transfer (Raymond, 2019). Due to their increased ability to survive in the host, resistant pathogens are likely to be transmitted within populations, particularly in a nosocomial setting (Raymond, 2019). As pathogens develop resistance mechanisms that render antibiotics ineffective, the ability to treat and prevent infection decreases, while patient mortality and the cost of healthcare increases (*Antibiotic resistance*, 2020; Tepekule et al., 2017).

Commensal organisms capable of harmlessly colonizing the human host form the majority of increasingly problematic species in terms of emerging drug resistance (Livermore et al., 2011; as cited by Raymond, 2019). These are organisms with distinct clinical significance defined by their dual nature to be harmless colonizers in some patients, and cause life-threatening hospital-acquired infections in others (Raymond, 2019). Gut commensals including *Enterococcus* spp. and *Enterobacteriaceae* (particularly *Escherichia coli* and *Klebsiella* spp.), and nasopharyngeal colonizer *Staphylococcus aureus* are predominating organisms within the context of AMR (Raymond, 2019). It is theorized that commensals predominate as antimicrobial resistant organisms due to experiencing selection pressures for resistance under a wider range of conditions compared to true pathogens, specifically during treatment for other infections (Raymond, 2019).

There are several factors contributing to the AMR crisis. Unsurprisingly, increased antibiotic use and decreased knowledge of AMR is strongly correlated with increased levels of resistant infections (Goossens et al., 2005, Grigoryan et al., 2007; as cited by Aminov, 2010). This stands to reason, given that antibiotic treatment inherently imposes a selection pressure upon bacteria in which it is to the benefit of their survival to develop resistance mechanisms. Compounding upon this, studies in developing countries such as India have revealed that major causative factors for AMR include poor public health infrastructure, a high burden of disease, widespread antibiotic use in animal farming, and especially the unregulated sale of cheap antibiotics (Laxminarayan & Chaudhury, 2016; as cited by Garvey et al., 2016). Likewise, in first world nations similar factors are pervasive. Poor antibiotic stewardship is largely to blame including unregulated non-therapeutic use of antimicrobials within the agricultural industry, poor patient compliance with antibiotic regimens, and poor prescribing practices (namely overreliance

on unsustainable monotherapy regimens, but also fulfilling patient requests for antibiotics is cases where they are not needed such as viral infections) (*Antimicrobial resistance*, n.d.; Raymond, 2019; Aminov, 2010).

Solutions to AMR

With so many factors contributing to the development of AMR, a multifaceted approach is required to address the AMR crisis (*Antimicrobial resistance*, n.d.; Aminov, 2010). A major component of this response will have to address the use of antibiotics in the human clinical setting. Of the many current approaches to prevent AMR, none have proved sufficient (Lomazzi et al., 2019; Raymond, 2019). A common solution proposed against AMR is to develop new antibiotics that pathogens have not developed resistance mechanisms to (Aminov, 2010). However new antibiotics are not being developed quickly enough to outpace AMR (Baker et al., 2018). Indeed, weaknesses in the drug development ecosystem are apparent considering that there have been no new classes of antibiotics identified since 1984 (Garvey et al., 2016). Thus, it is equally, if not more important, to use the available drugs in novel ways that prevent the emergence and spread of AMR (Baker et al., 2018; Lomazzi et al., 2019; Raymond, 2019; Tepekule et al., 2017). Scholars and global organizations alike are warning that even if new antibiotics are developed, without changing drug stewardship behaviors to reflect sustainable patterns of use, AMR will remain a major threat (*Antibiotic resistance*, 2020; Raymond, 2019).

Taking these recommendations into consideration, and given that a driving factor behind the development of resistance mechanisms is the practice of prescribing only a single class of antibiotic, some of the more promising methods to combat AMR are multidrug approaches that

focus on antibiotic heterogeneity (Masterton, 2010). Kollef (2006) describes antibiotic heterogeneity as the practice of utilizing multiple antibiotic classes to minimize AMR that might develop due to the selection pressures associated with using a limited number of antibiotic classes. Current multidrug solutions include antibiotic mixing, antibiotic cycling, and combination therapy. It is critical to note that all of these approaches are limited. A multitude of clinical trials and mathematical models exploring each method have provided inconclusive, conflicting results as to whether or not these approaches are truly effective against AMR (Beardmore et al., 2017; Brown & Nathwani, 2005; Kollef, 2006; Masterton, 2010; Raymond, 2019; Tepekule et al., 2017). In light of this, dosage based antibiotic cycling is a novel treatment approach that aims to combine the individual strengths of two current approaches, combination therapy and traditional antibiotic cycling, to synthesize an entirely new treatment method against AMR.

Combination Therapy

In combination therapy a patient is treated with multiple drugs from different antibiotic classes at once. In theory this prevents AMR due to antibiotic heterogeneity, and because it is very unlikely that a single microbe would simultaneously possess multiple mutations conferring drug resistance (Raymond, 2019). One complication associated with combination therapy as a proposed solution to AMR concerns the interactions of multiple drugs simultaneously, and whether drugs are acting independently, synergistically or antagonistically (Raymond, 2019). Furthermore, there are large concerns that the multidrug exposure characteristic of combination therapy can select for multidrug resistance in pathogens, in addition to the potential for adverse

pharmacological side effects in patients (Raymond, 2019). Despite this, studies have shown combination therapy to be particularly effective in preventing resistance in microbes where spontaneous mutations dominate the evolution of resistance, notably in the treatment of human immunodeficiency virus and tuberculosis (Monedero & Caminero, 2010, Vandamme & Camacho, 2011; as cited by Raymond, 2019). Furthermore, since patient-specific evolution conferring drug resistance occurs during antibiotic treatment, it has been suggested that individualized treatments may be more pertinent in addressing AMR (Mwangi et al., 2007, Blair et al., 2015; as cited by Beardmore et al., 2017). As a patient level approach, combination therapy is quite promising in this regard.

Traditional Antibiotic Cycling

Traditional antibiotic cycling is a population level approach that involves preferentially prescribing a certain class of antibiotic for a period of time, often many months, then switching to a different class, utilizing many cycles before the first drug is reintroduced. In theory this practice works because resistance imposes a fitness cost in the absence of antibiotic selection, thus resistance should decline when antibiotic use is suspended (Beardmore et al., 2017; Raymond, 2019). Research has emerged both in favor and against the use of antibiotic cycling as a treatment strategy to combat AMR (Tepekule et al., 2017). Afterall, antibiotic cycling will not work if resistance is not lost after a drug is withdrawn, or if resistance reemerges as soon as the drug is reinstated (Brown and Nathwani, 2005; Enne et al., 2001; as cited by Beardmore et al., 2017). Traditional antibiotic cycling is an appealing approach for proponents of antibiotic heterogeneity, however overall, there is no consensus in current literature concerning the

potential merits and risks of traditional antibiotic cycling. Studies have attributed the inconclusive results of this method to a variety of factors including the environment of implementation, cycling drugs of the same class, unstandardized cycling intervals, failure to repeat cycles, and confounding resistance management practices (Beardmore et al., 2017; Brown & Nathwani, 2005; Kollef, 2006).

Dosage Based Antibiotic Cycling

Cycling on a dosage interval is a novel approach that combines aspects of two current approaches, traditional antibiotic cycling and combination therapy. This new method is characterized by its use of extremely short cycling intervals, defined by the recommended dosage intervals of the medications used, that occur at the patient level. Like both aforementioned treatment strategies, dosage based antibiotic cycling is a multidrug treatment that promotes antibiotic heterogeneity. From combination therapy, dosage based antibiotic cycling incorporates treatment at the patient level. This method improves upon traditional antibiotic cycling by standardizing the cycling interval. It is notable that such short cycling intervals have never before been studied. The goal of this research is to explore the effectiveness of dosage based antibiotic cycling as a treatment option against AMR, by evaluating its ability to kill bacteria and minimize the development of antimicrobial resistance. This study utilizes strains of *S. aureus* and *E. coli* to ensure that the effects of dosage based antibiotic cycling are investigated within the context of both Gram positive and Gram negative organisms. *S. aureus* and *E. coli* were specifically chosen as the representative bacteria for a Gram positive and a Gram negative because both are extremely relevant and problematic organisms within the scope of drug resistant infections.

Methods

Kirby Bauer Disk Susceptibility Test for 10 Treatment Generations Using S. aureus and E. coli

Materials:

- *S. aureus* ATCC 25923
- *E. coli* ATCC 25922
- Blood agar plates
- Mueller Hinton agar plates
- Sterile forceps
- 30 µg Cefoxitin disks
- 2 µg Clindamycin disks
- 30 µg Ceftriaxone disks
- 10 µg Gentamicin disks

1. On the night before the Kirby Bauer Disk Susceptibility test is to begin, subculture the isolates onto separate blood agar plates such that one plate contains *S. aureus* and one plate contains *E. coli*. Incubate overnight.

2. For each isolate, create a 0.5 McFarland (McF) solution.

3. Lawn streak each 0.5 McF solution onto a Mueller Hinton agar plate. Using sterile forceps, place the appropriate antibiotic disk treatment onto each plate. For control, streak for growth each 0.5 McF solution onto a blood agar plate to ensure the purity and viability of each solution. Incubate each plate upside down for 16 hours.

    - Here and in subsequent steps, each Mueller Hinton plate may accommodate no more than two test groups.
    - In this experiment each tested isolate will have 3 test groups.
        - *S. aureus*

- Group 1 (Dosage based antibiotic cycling): Alternate treatment with 2 µg Clindamycin and 30 µg Cefoxitin every 16 hours.
- Group 2 (Cefoxitin Monotherapy): Receive 30 µg Cefoxitin every 16 hours.
- Group 3 (Clindamycin Monotherapy): Receive 2 µg Clindamycin every 16 hours.

- *E. coli*
  - Group 1 (Dosage based antibiotic cycling): Alternate treatment with 30 µg Ceftriaxone and 10 µg Gentamicin every 16 hours.
  - Group 2 (Ceftriaxone Monotherapy): Receive 30 µg Ceftriaxone every 16 hours.
  - Group 3 (Gentamicin Monotherapy): Receive 10 µg Gentamicin every 16 hours.

4. After incubation, measure the zone of inhibition for each test group and interpret using the Clinical & Laboratory Standards Institute criteria (see **Table 1**).

5. Using colonies from the edge of the zone of inhibition, create new 0.5 McF solutions corresponding to each test group.

6. Repeat Steps 3, 4, and 5 until 10 generations of treatment have passed.

Colony Biofilm Assay for 4 Treatment Generations Using S. aureus and E. coli

Note: This methodology has been adapted from that described by Merritt et al. in the July 2005 issue of *Current Protocols in Microbiology*.

Materials:

- *S. aureus* ATCC 25923
- *E. coli* ATCC 25922
- Blood agar plates
- Sterile forceps
- Vortex mixer
- 1X Sterile PBS
- Poretics 25mm black polycarbonate membranes with a pore size of 0.22 µm
- 15 mL sterile vortex tubes with tightly fitting lids
- 5 g lyophilized Oxacillin
- 10 mg lyophilized Clindamycin HCl
- 5 g lyophilized Ceftriaxone Sodium Salt Hemiheptahydrate
- 10 mg/mL Gentamicin Sulfate concentrate

1. Follow the manufacturer insert to dilute or reconstitute antibiotics to a concentration near the MIC value specific for the chosen isolates.

    - In this experiment the concentrations for each antibiotic were: 0.125 µg/mL Oxacillin, 0.06 µg/mL Clindamycin HCl, 1 µg/mL Ceftriaxone Sodium Salt Hemiheptahydrate, and 4 µg/mL Gentamicin Sulfate.

2. On the night before the Colony Biofilm Assay is to be begin, subculture the isolates onto separate blood agar plates such that one plate contains *S. aureus* and one plate contains *E. coli*. Incubate overnight to grow isolates to stationary phase.

3. Place five 25mm black polycarbonate membranes with a pore size of 0.22 µm shiny side up onto a sterile blood agar plate. Each test group will require its own plate with five membranes.

- In this experiment *S. aureus* and *E. coli* each have five test groups, requiring an initial set up with a total of 10 plates and 50 membranes (5 plates and 25 membranes for each isolate).
- The entire surface of the membrane must be in direct contact with the agar here and in all subsequent steps. Air bubbles may be removed by gently lifting the membrane and repositioning it on the plate.
- Leave plate covered whenever possible here and in all subsequent steps to maintain sterility of the membranes.

4. For each isolate make a 0.5 McFarland solution (1.5×10^8 cfu/mL). Inoculate each membrane with 5 μL of diluted culture. When the liquid has dried incubate plates upright for 24 hours.

5. Using sterile forceps, gently lift each membrane off of the plate and transfer to a fresh agar plate. Incubate plates upright for 24 hours.

- Following this step, the colony biofilms will have been grown for a total of 48 hours. Every 24 hours the membranes will now be transferred to new plates to expose the colony biofilms to a fresh nutrient supply and antimicrobial treatment according to the designated test group.

6. After the incubation is complete, sample biofilm growth.

- Aseptically transfer one membrane from each plate to separate 15 mL tubes containing 10 mL sterile 1X PBS such that each tube contains a single membrane.
    - The membrane can be gently folded on itself before it is lifted off the plate to allow it to fit more easily into the 15 mL tube.

- Cap tubes and vortex samples for two pulses of 60 seconds each to detach all bacteria, creating a homogenous suspension.
- Prepare a dilution series of the vortexed samples by performing serial dilutions of the vortexed suspension, using PBS as diluent. Final dilution factors are 1:10,000, 1:100,000, and 1:1,000,000. Streak 100 µL of each dilution on a separate blood agar plate, incubating overnight to determine the average number of colony forming units (cfu) per mL recovered from each membrane.
    - The cfu counts obtained here will serve as the baseline for future experimentation, this sampling point is designated T = 0 with respect to the start of experimental antimicrobial treatments. With each subsequent generation of treatment T = 1, 2, 3, 4 accordingly.
    - cfu/mL is calculated by $\frac{(number\ of\ colonies\ counted)(dilution\ factor)}{volume\ quantified}$

7. Transfer the remaining membranes from each plate to fresh agar plates, inoculating the membranes with the antibiotic treatment appropriate for the test group. For each membrane, administer the antibiotic by pipetting 30 µL of the appropriate antibiotic solution onto the surface of the agar such that it is a singular drop. Center the membrane over the drop and gently place the membrane directly over it. This causes the antibiotic to fully and evenly disperse between the surfaces of the agar plate and the membrane as the membrane comes to rest (see **Figure 1**). Incubate upright 24 hours.

- In this experiment each tested isolate will have 5 test groups.
    - *S. aureus*

- Group 1 (Dosage based antibiotic cycling): Alternate treatment with 0.125 µg/mL Oxacillin and 0.06 µg/mL Clindamycin HCl every 24 hours.
- Group 2 (Oxacillin Monotherapy): Receive 0.125 µg/mL Oxacillin every 24 hours.
- Group 3 (Clindamycin Monotherapy): Receive 0.06 µg/mL Clindamycin HCl every 24 hours.
- Group 4 (Combination Therapy) Receive 0.125 µg/mL Oxacillin and 0.06 µg/mL Clindamycin HCl simultaneously every 24 hours. (administered as a solution containing equal parts of both drugs)
- Group 5 (Control): Receive no treatment; verifies purity of test conditions.

- *E. coli*
  - Group 1 (Dosage based antibiotic cycling): Alternate treatment with 1 µg/mL Ceftriaxone Sodium Salt Hemiheptahydrate and 4 µg/mL Gentamicin Sulfate every 24 hours.
  - Group 2 (Ceftriaxone Monotherapy): Receive 1 µg/mL Ceftriaxone Sodium Salt Hemiheptahydrate every 24 hours.
  - Group 3 (Gentamicin Monotherapy): Receive 4 µg/mL Gentamicin Sulfate every 24 hours.
  - Group 4 (Combination Therapy) Receive 1 µg/mL Ceftriaxone Sodium Salt Hemiheptahydrate and 4 µg/mL Gentamicin Sulfate

simultaneously every 24 hours (administered as a solution containing equal parts of both drugs).

- Group 5 (Control): Receive no treatment; verifies purity of test conditions.

8. When incubation is complete, remove 1 membrane from each plate and quantitate biofilm growth as in Step 6. Transfer the remaining membranes to new plates and continue appropriate treatment and incubation.

9. Repeat Step 8 until all colony biofilms have been sampled.

Results

S. aureus Kirby Bauer Susceptibility

It is recommended to reference the data shown in **Table 2** and **Figure 2** to better interpret the following results. Monotherapy treatment with 30 µg Cefoxitin did not induce resistance in the *S. aureus* isolate, with the final zone of inhibition measuring 23 mm. Monotherapy treatment with 2 µg Clindamycin did not induce resistance in the *S. aureus* strain, with the final zone of inhibition measuring 22 mm. Dosage based antibiotic cycling, alternating with 30 µg Cefoxitin and 2 µg Clindamycin, induced intermediate resistance in the *S. aureus* isolate overall, with the final zone of inhibition measuring 18 mm.

E. coli Kirby Bauer Susceptibility

It is recommended to reference the data shown in **Table 3** and **Figure 3** to better interpret the following results. Monotherapy treatment with 30 µg Ceftriaxone did not induce resistance in the *E. coli* isolate, with the final zone of inhibition measuring 28 mm. Results from Generation 4 were deemed unreliable data as there was adequate growth on the test plate, but no growth on the control plate. Results from Generation 6 are deemed unreliable due to poor growth on the test plate despite adequate growth on the control plate. Monotherapy treatment with 10 µg Gentamicin induced intermediate resistance in the *E. coli* isolate, with the final zone of inhibition measuring 14 mm. Dosage based antibiotic cycling, alternating with 30 µg Ceftriaxone and 10 µg Gentamicin, did not induce resistance in the *E. coli* strain, with the final zone of inhibition measuring 19 mm. Results from Generation 4 and 8 are deemed as unreliable data as there was poor growth on the test plate despite adequate growth on the control plate.

S. aureus Colony Biofilm Assay

It is recommended to reference the data shown in **Table 4** and **Figure 4** to better interpret the following results. Monotherapy treatment with 0.125 µg/mL Oxacillin against *S. aureus* produced a trend of decreasing cfu/mL. Cfu/mL results from Generation 0 were calculated to be 14.2×10^8, and had decreased to 8.7×10^8 by Generation 4. Monotherapy treatment with 0.06 µg/mL Clindamycin against *S. aureus* produced a steady trend of cfu/mL counts with no significant increase or decrease. Data from Generation 3 was not reported as the colony count exceeded the range of accuracy (>300 colonies). Combination therapy with 0.125 µg/mL Oxacillin and 0.06 µg/mL Clindamycin against *S. aureus* produced an overall decreasing trend in cfu/mL counts. Cfu/mL results from Generation 0 were calculated to be 21.7×10^8, and had decreased to 5.1×10^8 by Generation 4. Dosage based antibiotic cycling treatment, alternating

with 0.125 µg/mL Oxacillin and 0.06 µg/mL Clindamycin, produced a trend of increasing cfu/mL. Cfu/mL results from Generation 1 was calculated to be 4.4 x 10^8, and had increased to 8.9 x 10^8 by Generation 4. Data from Generation 0 was not reported as the colony count exceeded the range of accuracy (>300 colonies).

E. coli Colony Biofilm Assay

It is recommended to reference the data shown in **Table 5** and **Figure 5** to better interpret the following results. Monotherapy treatment with 1 µg/mL Ceftriaxone against *E. coli* induced widely varied results for calculated cfu/mL. The lowest cfu/mL count was from Generation 1 and was calculated to be 4.2 x 10^8, while the highest cfu/mL result was recorded from Generation 2 at 20.7 x 10^8. Monotherapy treatment with 4 µg/mL Gentamicin against *E. coli* produced slightly varied results for calculated cfu/mL, with an overall increasing trend. Cfu/mL counts from Generation 0 began at 6.5 x 10^8, peaked at Generation 2 with 25.3x 10^8, and ended on Generation 3 with 8.9 x 10^8. Data from Generation 4 was not reported as the colony count exceeded the range of accuracy (>300 colonies). Combination therapy with 1 µg/mL Ceftriaxone and 4 µg/mL Gentamicin produced a trend of steadily increasing cfu/mL counts. Cfu/mL results from Generation 0 was calculated to be 5.1 x 10^8, and had increased to 26.5 x 10^8 by Generation 4. Dosage based antibiotic cycling treatment, alternating with 1 µg/mL Ceftriaxone and 4 µg/mL Gentamicin, reveal a trend of increasing cfu/mL counts. Cfu/mL results from Generation 0 were calculated to be 5.8 x 10^8, and had increased to 18.8 x 10^8 by Generation 4.

Discussion

Kirby Bauer Susceptibility for S. aureus and E. coli

Comparing the effectiveness of monotherapy and dosage based antibiotic cycling in preventing the development AMR using Kirby Bauer Disk Susceptibility was ultimately inconclusive. In some groups for example, Clindamycin monotherapy against *S. aureus* and Ceftriaxone monotherapy against *E. coli*, results strongly indicated persisting antibiotic susceptibility and the prevention of AMR development in the involved bacterial strains. In both cases there was a slight trend progressing towards intermediate and full drug resistance, however it is likely it would have taken numerous additional generations to induce drug resistance in the isolate. Significantly, this does not reflect upon the development of true AMR because prolonged drug exposure always induces drug resistance given enough time. These results would suggest that the monotherapy treatment did not contribute to the development of AMR.

Other trials had different outcomes. The results from the group with Cefoxitin monotherapy against *S. aureus*, and Gentamicin monotherapy against *E. coli* would suggest that the use of monotherapy treatment did contribute to the development of AMR. This is notable in the induced intermediate resistance status observed in the *E. coli* isolates receiving Gentamicin monotherapy throughout Generations 6-10. While the Cefoxitin monotherapy treatment did not induce AMR as observed in the ten generation timeframe of this experiment, it can be reasonable predicted that provided only a few more treatment generations, this monotherapy treatment would have also induced full drug resistance. This predicted outcome is evident by the sharp trend of decreasing drug susceptibility observed in this trial.

Kirby Bauer Disk Susceptibility results for dosage based antibiotic cycling were quite intriguing. Upon first observation, one can note that when used against the *S. aureus* strain this treatment method induced intermediate drug resistance, yet did not induce AMR when used

against *E. coli*. Upon closer inspection of the data a more compelling trend emerges however. In both instances, generations exposed to one of the drugs experienced hardly any change in drug susceptibility throughout the course of the experiment, while generations exposed to the alternate drug experienced decreasing drug susceptibility. With *S. aureus*, strong antibiotic susceptibility was maintained in generations exposed to the first drug introduced (30 ug Cefoxitin), and was lost to progressive intermediate resistance during generations exposed to the second drug cycled (2 ug Clindamycin). Meanwhile the opposite occurred with the *E. coli* isolate as susceptibility decreased in generations exposed to the first drug (30 ug Ceftriaxone), yet generations exposed to the second drug cycled (10 ug Gentamicin) maintained strong antibiotic susceptibility.

The Kirby Bauer Disk Susceptibility method was selected for use in this research due to its ability to quantitatively measure the resistance status of a specific isolate over time. It is an excellent methodology to measure and compare the ability of different treatment methods to prevent the development of AMR. The inconclusiveness of the results attained via this method may in part be attributed to outside sources of error occurring throughout the process of this methodology. Notable is the limitations imposed by unavoidable human technical error. Lacking access to an instrument that measures the precise optical density of a prepared McF solution, the concentration of the McF solutions prepared in the course of this research were not uniformly standardized. Working McF solutions were estimated to be approximately the optical density of a 0.5 McF solution by visually comparing them by eye to a manufactured 0.5 McF standard. This carries over to cause variations in dissolved colony concentration in the McF solutions, contributing to become a significant source of error in this research. Precision of the working McF solutions is critical in a repeated generation Kirby Bauer assay to ensure that experimental conditions (i.e., the concentration of bacteria inoculated onto the plate) is the same in each trial.

In addition to this source of error, some of the blood agar plates utilized during the course of research were expired due to resource acquisition limitations imposed by the Covid-19 pandemic. This, combined with the human error described above, likely explains the inadequate growth observed on the test plates in Generations 4 and 8 of Gentamicin monotherapy and Generation 6 of Ceftriaxone monotherapy, as well as the inadequate growth on the control plate observed in Generation 4 of Ceftriaxone monotherapy.

An additional factor to consider is that for biosafety reasons, this research was conducted using ATCC organisms which are known to be stable. These ATCC strains of bacteria do not accurately represent how wild strains of bacteria would behave when exposed to the same testing conditions and thus interferes with the conclusiveness of the results from the Kirby Bauer Disk Susceptibility test. It is likely that repeating this testing methodology using wild strains of bacteria that more readily mutate and acquire mechanisms for drug resistance could potentially alter the outcome of the research, and would be a truer reflection of the bacterial response to these treatment methods in a clinical setting. This combination of factors contributes to the overall inconclusiveness of the results recorded during this methodology. Variations in the nutrient status of agar plates, combined with a non-standardized concentration of pathogen inoculated onto the membrane for each trial undermines the validity of the data recorded and interferes with the ability to draw concise conclusions.

Colony Biofilm Assay for S. aureus and E. coli

The Colony Biofilm Assay technique was used in addition to Kirby Bauer Disk Susceptibility because unlike the Kirby Bauer method, it can quantitate growth over time, and

has been found to be especially useful in studying the antibiotic resistant properties of bacteria (Merritt et al., 2005). Through colony count quantification the Colony Biofilm Assay can qualitatively measure the resistance status of a specific isolate over time by exploiting the biofilm formation of the bacterial isolates. The thickness of the biofilm formed in each group is reflective of the treatment's ability to prevent the development of AMR since biofilms display increased antibiotic tolerance compared to planktonic cells; a thicker biofilm (many cfu/mL) indicates a more drug resistant pathogen while a thinner biofilm (few cfu/mL) indicates drug susceptibility (Hussey et al., 2017).

Comparing the ability of monotherapy, combination therapy, and dosage based antibiotic cycling to prevent the development of AMR using a Colony Biofilm Assay ultimately produced inconclusive results. With *S. aureus*, the only treatment group to experience a definitive increasing trend in cfu/mL results was the group receiving dosage based antibiotic cycling. This would suggest that this treatment method was not successful in preventing the development of AMR. The treatment group receiving Clindamycin monotherapy did not experience much variation in colony counts over time, suggesting that drug susceptibility was maintained. Likewise, the groups receiving combination therapy and Oxacillin monotherapy actually experienced a trend of decreasing cfu/mL results. This is suggestive of the ability to not only maintain drug susceptibility, but also prevent any further growth of the biofilm. Overall, evaluating the results of the *S. aureus* Colony Biofilm Assay, it can be concluded that dosage based antibiotic cycling was the least effective strategy in preventing the development of AMR, and might have even contributed to its progression. Meanwhile with the *E. coli* trials, monotherapy, combination therapy, and dosage based antibiotic cycling treatment all resulted in an increasing trend of expanding colony counts. This would suggest that unlike what occurred

with *S. aureus*, in this case all treatment methods were equally unable to prevent the development of AMR.

Overall, no definitive conclusions can be drawn regarding the ability of these treatment methods to prevent or promote AMR, or concerning which of these treatment methods is superior or inferior in that regard compared to the others. Taking into consideration that both organisms experienced increasing cfu/mL results with dosage based antibiotic cycling, it is possible that it may have contributed to the development of AMR rather than prevented it, however in light of profound limitations to the research methodology there is no way to be certain. The nature of the Colony Biofilm Assay methodology is to form an initial biofilm of the organism and then subject that biofilm to experimental conditions. Conducting this experiment, it came to pass that vortexing the biofilm growth on each membrane for 2 minutes could not fully dissolve all bacteria into solution. Vortexing for longer than the recommended amount of time might have been able to fully suspend the biofilm growth in solution, but this was not performed as doing so can rupture and kill the organism resulting in no growth quantification whatsoever.

This resulted in a distinct source of error: quantitation suspensions that were not fully representative of the true concentration of viable bacteria forming the biofilm. Furthermore, there was no way to standardize this variation. One suspension may have contained 85% of the biofilm, while another contained 70% of the biofilm, etc… Compounding upon this was the possibility for an undissolved piece of biofilm to be aspirated into the pipette tip when sampling to streak for cfu/mL quantification. This would cause a false increase in the bacteria recovered that is not representative of the concentration dissolved into solution in the suspension. This source of error is reflected in the variation of the calculated cfu/mL results for each trial. It also helps to explain why Generation 0 of dosage cycling with *S. aureus*, Generation 3 of

Clindamycin monotherapy with *S. aureus*, Generations 2 and 3 of the *S. aureus* control group, Generation 4 of Gentamicin monotherapy with *E. coli*, and Generation 2 of the *E. coli* control group do not have data recorded due to colony counts exceeding the limit of accuracy. Despite streaking for growth at three different dilution factor levels, some trials experienced such a vast degree of variation that accurate levels of colony growth were not able to be recovered.

In addition to this source of error, some of the blood agar plates utilized during the course of research were expired due to resource acquisition limitations imposed by the Covid-19 pandemic. This resource limitation is also the reason that the Colony Biofilm Assay methodology was only able to evaluate 4 generations of treatment, as opposed to a more appropriate duration of experimentation that would be closer to 10 generations. Furthermore, as described above, this research was also conducted using ATCC organisms which are known to be stable. These ATCC strains of bacteria do not accurately represent how wild strains of bacteria would behave when exposed to the same testing conditions and thus interferes with the conclusiveness of the results from the Colony Biofilm Assay. It is likely that repeating this testing methodology using wild strains of bacteria that more readily mutate and acquire mechanisms for drug resistance could potentially alter the research outcome, and would be a more accurate reflection of the bacterial response to these treatment methods in a clinical setting.

Resource limitation and use of stable bacteria strains, combined with the suspension error described above, contribute to the overall inconclusiveness of the results recorded during the Colony Biofilm Assay. Relatively short experiment duration, variations in the nutrient status of agar plates, and non-standardized vortex suspensions are factors that each undermine the validity of the data recorded and interferes with the ability to draw concise conclusions.

Conclusion

The combined results of both methodologies performed in this experiment are not conclusive as to whether dosage based antibiotic cycling is able to prevent AMR in general, or whether it is more or less effective than current treatment approaches such as monotherapy and combination therapy. Traditional antibiotic cycling, an existing method against AMR that likewise utilizes antibiotic heterogeneity was not evaluated in this study due to its status as a population level approach. As this research only utilized in vitro testing methods that are representative of patient level approaches, purely independent from patient context, this study was unable to evaluate traditional antibiotic cycling. Comparison of dosage based antibiotic cycling with traditional antibiotic cycling may better fit the scope of a future study.

This research both proposes and investigates a novel treatment approach to prevent the development of AMR. While the results of this particular study are inconclusive, it is recommended that further research be performed. To improve upon the limitations hindering this study, future research should ensure standardization of test conditions, and conduct research for several generations of bacterial growth using wild strains. An anticipated critique of dosage based antibiotic cycling will be in its hypothetical implementation should it ever be approved as a treatment method for patient use. It is noted in literature surrounding the issue of AMR that treatment options that involve changing the antibiotic regimen too frequently can create problems with implementation and patient compliance (Raymond, 2019). In a nosocomial inpatient setting where antibiotics are administered by health professionals anyways, it is not anticipated that this will be a large issue. Ease of patient compliance can be encouraged in patients receiving dosage cycled antibiotics over the counter by packaging antibiotics in an easy-

to-follow blister pack, similar to how medication regimens with doses that vary in concentration and/or composition are already administered today.

Dosage based antibiotic cycling is a novel suggested treatment method for preventing the emergence of AMR in patient infections. Given that new antibiotics are not being discovered rapidly enough to keep up with progressively evolving resistance mechanisms, it is absolutely necessary to explore using current antibiotics in new and inventive ways. Dosage based antibiotic cycling is a new theory that leaves much room for exploration. The effect of different drug concentrations, utilizing different strains and/or species of bacteria, and utilizing dose cycles of different drug parings are all potential avenues that further research would do well to explore. This study only cycled two drugs at a time, however, dosage based antibiotic cycling could theoretically allow three or even four drugs to be cycled in the same patient, provided there are no adverse pharmacological side effects. Despite the inconclusive results presented in this study, dosage based antibiotic cycling may yet prove to be a useful tool in the multifaceted approach that will be required if we wish to fully address and resolve the AMR crisis.

Conflict of Interest Statement

The author declares no conflict of interest.

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Appendix

Table 1:

Relevant Clinical & Laboratory Standards Institute Criteria				
Organism	Disk content	Zone Diameter Breakpoints (mm)		
		Susceptible	Intermediate	Resistant
S. aureus	2 µg Clindamycin	≥21	15-20	≤14
	30 µg Cefoxitin	≥22		≤21
E. coli	30 µg Ceftriaxone	≥23	20-22	≤19
	10 µg Gentamicin	≥15	13-14	≤12

Table 2:

S. aureus Kirby Bauer Disk Susceptibility Testing Zone of Inhibition											
		Generation									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment	Monotherapy 30 µg Cefoxitin	29 mm	26 mm	24 mm	23 mm	25 mm	24 mm	24 mm	25 mm	23 mm	23 mm
	Dosage Cycling 30 µg Cefoxitin (Generation: 1, 3, 5, 7, 9) 2 µg Clindamycin (Generation: 2, 4, 6, 8, 10)	29 mm	21 mm	30 mm	19 mm	29 mm	18 mm	30 mm	19 mm	29 mm	18 mm
	Monotherapy 2 ug Clindamycin	24 mm	27 mm	23 mm	23 mm	24 mm	22 mm	23 mm	22 mm	23 mm	22 mm

Table 3:

E. coli Kirby Bauer Disk Susceptibility Testing Zone of Inhibition											
		Generation									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Treatment	Monotherapy 30 µg Ceftriaxone	32 mm	32 mm	33 mm	30 mm**	29 mm	31 mm*	24 mm	31 mm	27 mm	28 mm
	Dosage Cycling 30 µg Ceftriaxone (Generation: 1, 3, 5, 7, 9) 10 µg Gentamicin (Generation: 2, 4, 6, 8, 10)	33 mm	19 mm	34 mm	21 mm*	33 mm	20 mm	27 mm	22 mm*	28 mm	19 mm
	Monotherapy 10 µg Gentamicin	19 mm	20 mm	18 mm	19 mm	16 mm	13 mm	13 mm	13 mm	13 mm	14 mm

*: Data point unreliable (Adequate growth on control plate, poor growth on test plate)

**: Data point unreliable (No growth on control plate, adequate growth on test plate)

Table 4:

S. aureus Colony Biofilm Assay cfu/mL x 10^8						
		Generation				
		0	**1**	**2**	**3**	**4**
Treatment	Dosage Cycling 0.125 µg/mL Oxacillin (Generation: 1, 3) 0.06 µg/mL Clindamycin (Generation: 2, 4)	*	4.4	6.7	7.6	8.9
	Monotherapy 0.125 µg/mL Oxacillin	14.2	4.5	5.9	10.8	8.7
	Monotherapy 0.06 µg/mL Clindamycin	9.2	3.9	7.1	*	10.0
	Combination Therapy 0.125 µg/mL Oxacillin and 0.06 µg/mL Clindamycin	21.7	9.3	7.0	8.8	5.1
	Control	12.7	7.5	*	*	9.7

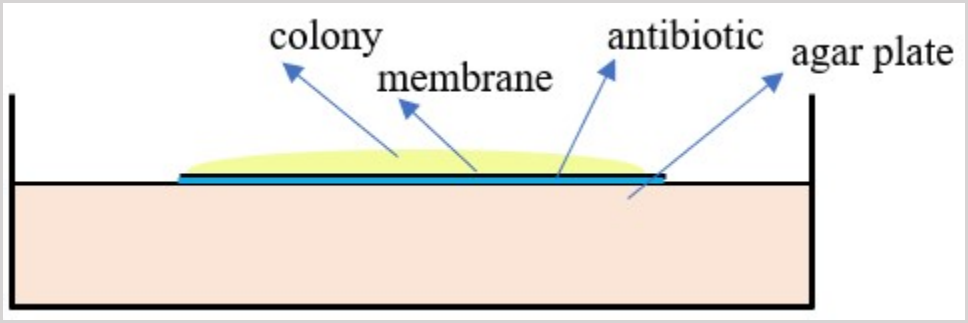
*: Data not reported, colony count above range of accuracy for calculating cfu/mL (count greater than 300)

Table 5:

E. coli Colony Biofilm Assay cfu/mL x 10^8						
		Generation				
		0	**1**	**2**	**3**	**4**
Treatment	Dosage Cycling 1 µg/mL Ceftriaxone (Generation: 1, 3) 4 µg/mL Gentamicin (Generation: 2, 4)	5.8	4.5	12.5	22.9	18.8
	Monotherapy 1 µg/mL Ceftriaxone	8.8	4.2	20.7	12.0	10.4
	Monotherapy 4 µg/mL Gentamicin	6.5	5.0	25.3	8.9	*
	Combination Therapy 1 µg/mL Ceftriaxone and 4 µg/mL Gentamicin	5.1	6.5	8.9	29.3	26.5
	Control	28.2	3.3	*	15.6	12.6

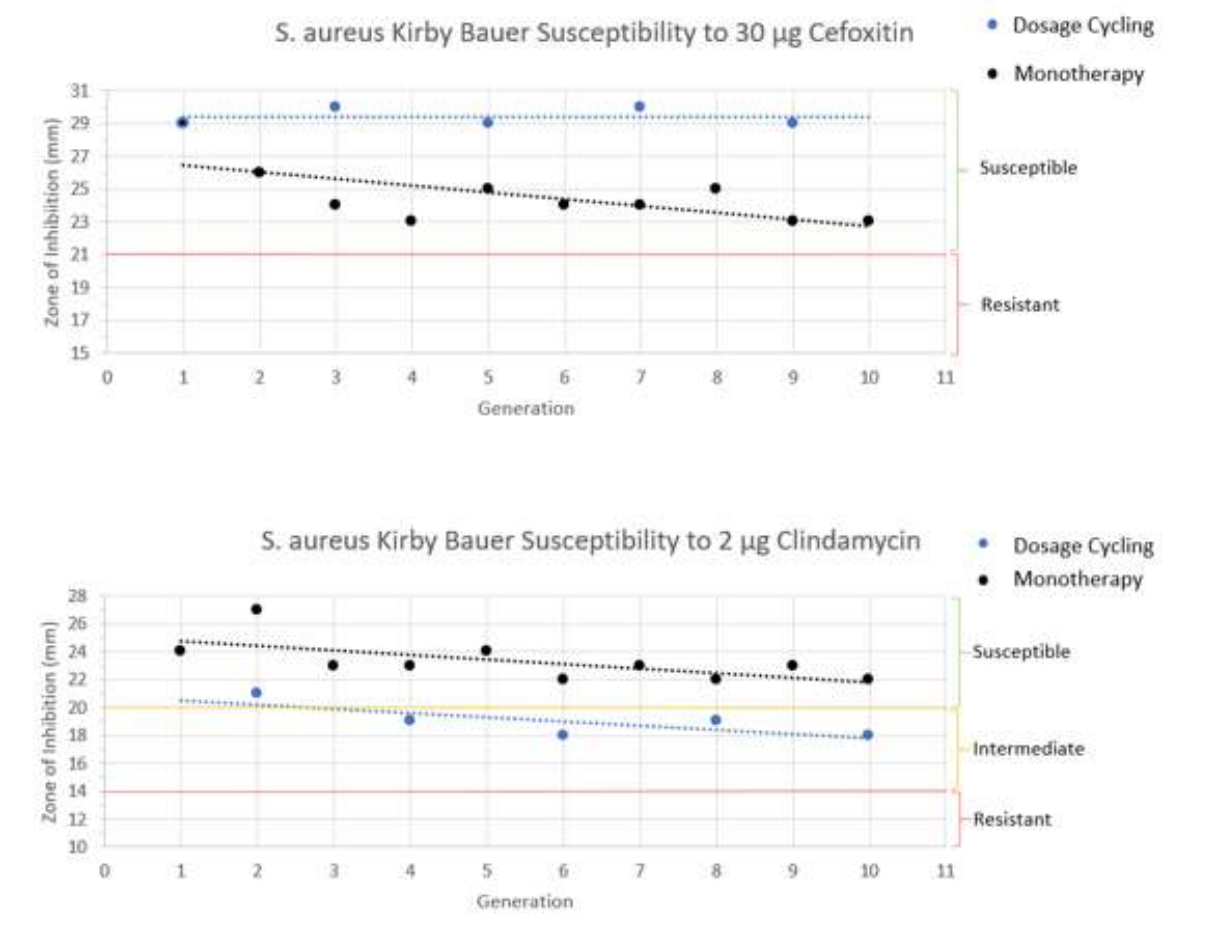
*: Data not reported, colony count above range of accuracy for calculating cfu/mL (count greater than 300)

Figure 1:



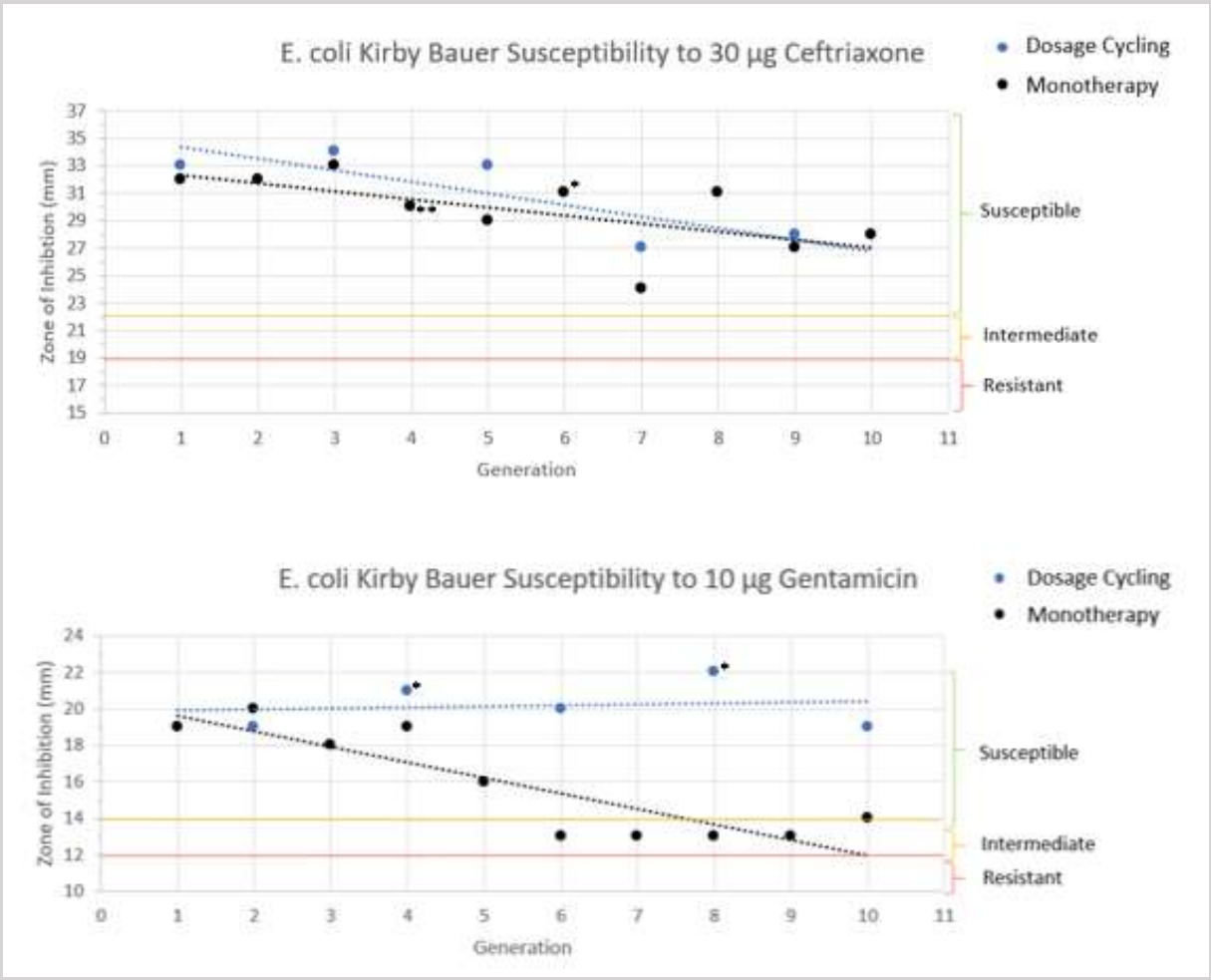
Set up for a Colony Biofilm Assay (depiction shows a single membrane on the agar plate, however up to 6 membranes can fit on each plate).

Figure 2:



When interpreting Kirby Bauer Susceptibility data for dosage based antibiotic cycling, consider that odd numbered generations of *S. aureus* received Cefoxitin (top), while even numbered generations received Clindamycin (bottom), such that both graphs should be interpreted as a single figure. Note how *S. aureus* maintains strong antibiotic susceptibility with generations receiving dosage based antibiotic cycling with Cefoxitin, yet progressively develops intermediate drug resistance with generations receiving dosage based antibiotic cycling with Clindamycin.

Figure 3:

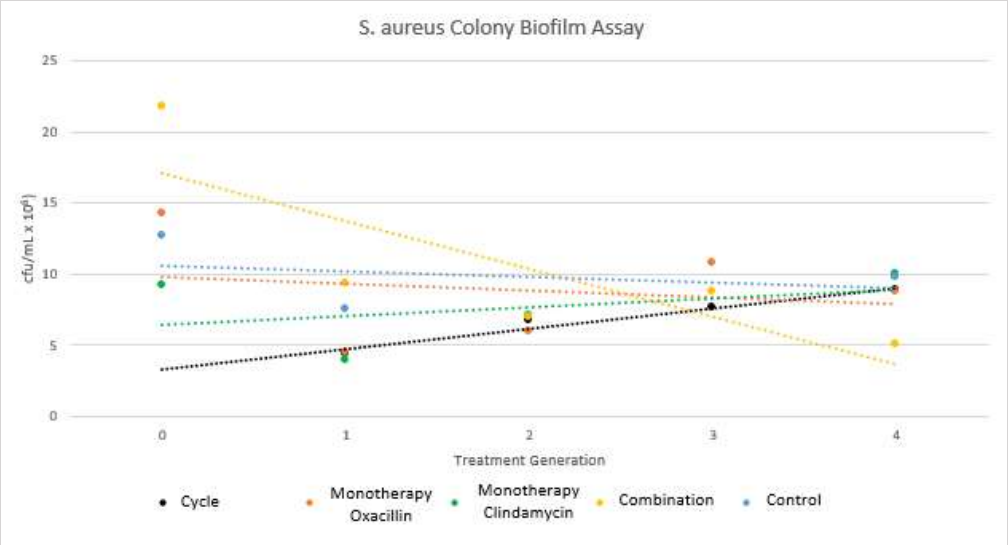


When interpreting Kirby Bauer Susceptibility data for dosage based antibiotic cycling, consider that odd numbered generations of *E. coli* received Ceftriaxone (top), while even numbered generations received Gentamicin (bottom), such that both graphs should be interpreted as a single figure. Note how *E. coli* maintains strong antibiotic susceptibility with generations receiving dosage based antibiotic cycling with Gentamicin, yet experiences decreasing drug susceptibility with generations receiving dosage based antibiotic cycling with Ceftriaxone.

*: Data point unreliable (Adequate growth on control plate, poor growth on test plate)

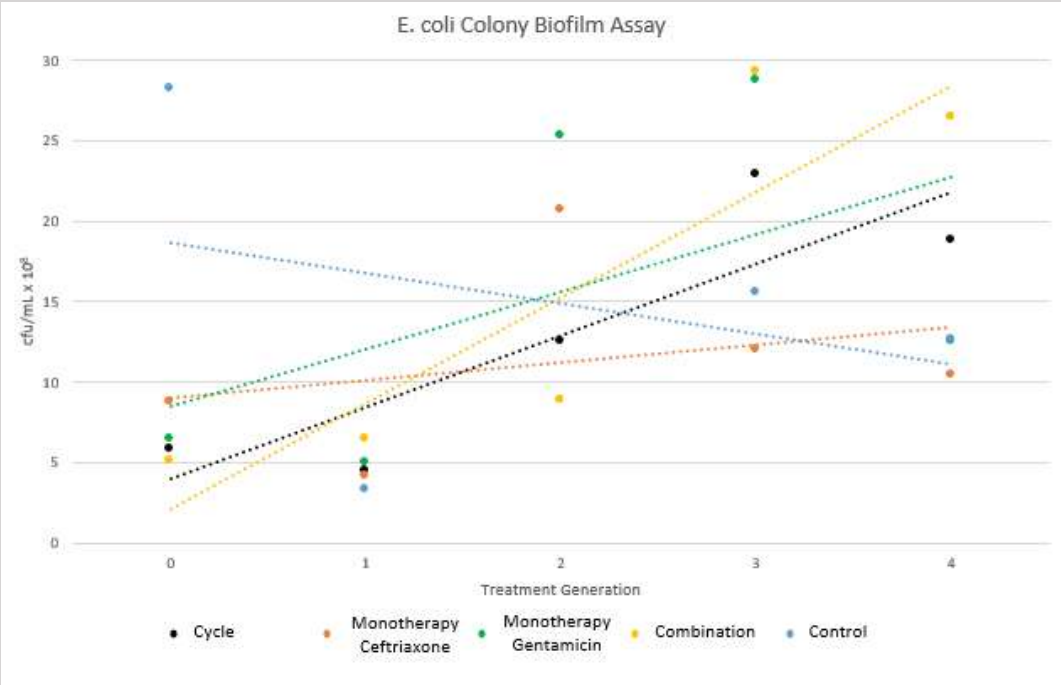
**: Data point unreliable (No growth on control plate, adequate growth on test plate)

Figure 4:



With *S. aureus* it is clear to see that the only treatment method that appears to effectively prevent the development of AMR is combination therapy. In this case dosage based antibiotic cycling may have actually contributed to the development of AMR as suggested by the slightly upward trend in cfu/mL counts observed with this group.

Figure 5:



Individual data points express a wide degree of variation due to errors discussed in detail above. By fitting a trendline to the data it is possible to better interpret the results, with the consistent upward trend in cfu/mL counts revealing that all treatment methods were equally unable to prevent the development of AMR in *E. coli.*