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The Impact of Antibiotic Exposure and Concentration on Resistance in Bacteria

Ahmad G. Abdel-Azim[1] and Gamal A. Abdel-Azim[2, 3]

[1]Classical Charter School, Appleton, WI

[2]International Center for Biotechnology, Verona, WI

[3]Cooperative Resources International, Verona, WI

Summary

How many generations are needed for bacteria to gain antibiotic resistance and to what extent does antibiotic concentration make a difference? To address these questions, pure *Escherichia coli* was cultured on nutrient agar in the presence of low, medium, and high concentrations of amoxicillin. Surviving bacteria were repeatedly cultured for four consecutive generations, and the sensitivity of bacteria was measured within each generation. We measured antibiotic sensitivity by evaluating the diameter of the inhibition zone around antibiotic-saturated discs of all three amoxicillin concentrations. Although exposing bacteria to extremely heavy antibiotic concentrations resulted in greater inhibition zones around antibiotic-saturated discs, there were no major differences in sensitivity relative to the much lower concentrations studied. We also found that bacteria gained significant resistance upon first exposure to the antibiotic. In fact, the reduction in average inhibition zones was much greater in early generations than in late generations. We conclude that *E. coli* can gain major resistance upon surviving early exposure to amoxicillin, which suggests that antibiotic courses of medication should be prescribed to completely clear the body of bacterial infection upon first antibiotic exposure.

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Introduction

The advent of antibiotics in the 20th century has rendered many formerly dangerous diseases easily treatable. However, as more and more antibiotics are used worldwide, bacteria are becoming resistant to antibiotic treatments. Antibiotic-resistant bacteria are not only a threat to humans but also to animals. Alexander Fleming, who discovered the first antibiotic, predicted that the misuse of penicillin may lead to mutant forms of bacteria that could resist the drug (1). This prediction has not only become a reality for penicillin, but for other antibiotics as well. Even before antibiotics were first used to treat infections, resistant organisms had been isolated (2).

In antibiotic-rich environments, such as hospitals, antibiotic resistance occurs rapidly due to the eradication of weaker (non-resistant) bacteria. As this process of eradication continues, the more resistant bacteria thrive. When they proliferate multiple times, a resistant strain of bacteria is created (3).

Antibiotics are synthesized from chemicals or occur naturally. They neutralize bacteria by suspending cell wall synthesis or interfering with a vital process, such as protein synthesis, while leaving human cells unaffected (4). For example, amoxicillin, which belongs to the penicillin group, prevents bacteria from forming cell walls; consequently, bacteria cease to grow and eventually die (5). However, bacteria are versatile organisms that mutate at a high rate. A mutation may give a bacterium the ability to neutralize an antibiotic before it takes effect or to expel any antibiotic molecules that manage to penetrate the bacterium's cell membrane (6).

Bacteria can also gain resistance to an antibiotic by receiving a resistance gene from another bacterium through conjugation, a process in which bacteria bond through pili and exchange genes. A bacterium can also release its DNA into the environment upon death to be picked up by another bacterium (6).

Currently, there are numerous strains of resistant bacteria. For example, *Salmonella* gains resistance to beta-lactam antibiotics by producing an enzyme called beta-lactamase. This enzyme attacks the beta-lactam ring, leaving the antibiotic obsolete (3). *Escherichia coli*, a diverse group of bacteria with resistant strains that cause diarrhea and kidney failure, can prevent the function of antibiotics by expelling any molecules that manage to enter through the cell wall (3). More specifically, when an antibiotic enters a resistant *E. coli*, the antibiotic is recognized and pumped out by the efflux pump, expelling any antibiotic that manages to penetrate the cell membrane (7).

As the number of effective antibiotics decreases, there is a need to understand the factors that promote

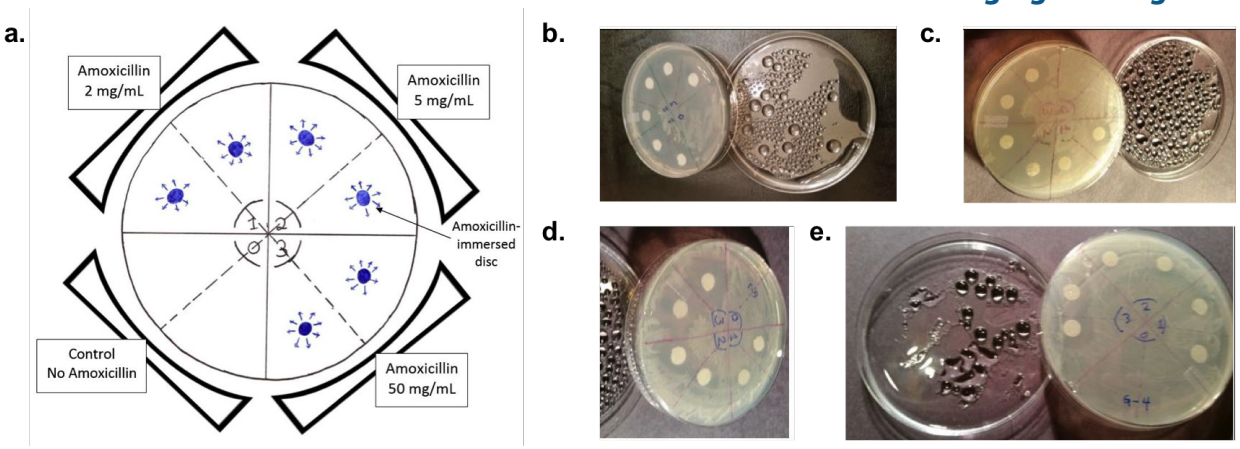

Figure 1: Petri dishes. (a) Diagram of experimental design. We developed a novel experimental design to test multiple amoxicillin concentrations. Two discs of each of three amoxicillin concentrations were placed in a quarter. A control, untreated quarter was left in each plate. (b) Generation 1. *E. coli* culture growth after 48 hours of incubation at 37° C. Dishes were incubated upside down to keep condensation off the culture. (c) Generation 2. Culture grew completely in section 0 with no antibiotic discs and partially in section 1 with low antibiotic concentration. Other sections had minor growth far away from medicated discs. (d) Generation 3. Culture grew in all sections but with larger inhibition zones around discs with greater antibiotic concentrations. (e) Generation 4. Slight inhibition zones were observed around highly concentrated discs with 50 mg/mL. *E. coli* in sections with lower antibiotic concentrations grew all the way to medicated discs in defiance of the antibiotic.

antibiotic resistance in order to find viable solutions to the problem. In this experiment, we studied the impact of exposing *E. coli* to amoxicillin at a wide range of concentrations. We examined the path and rate at which bacteria gain resistance by exposing them to the same antibiotic for several generations. We hypothesized that increased exposure and elevated dosage promote amoxicillin resistance in *E. coli*.

Results

In each generation, 12 observations were recorded from two Petri dishes and were listed in the table according to their corresponding disc and concentration. This experiment was designed to allow 4 replicates per concentration in each generation. The greatest susceptibility of *E. coli* to amoxicillin occurred in generation 1. In the final generation, little or no inhibition of bacterial growth was observed in all sections except for section 3, which had highly concentrated discs (**Figure 1b-e**). The radius of the inhibition zone dropped from an overall average of 1.49 cm in generation 1 to 0.53 cm in generation 4 (**Table 1**), following a quadratic curve (**Figure 2a**). In general, more concentrated antibiotic solutions resulted in greater inhibition zones within each of the 4 generations studied, producing a linear relationship (**Figure 2b**). Statistical analysis to confirm these observations is reported in the statistical

	Generation			
Amoxicillin (cm/mL)	1	2	3	4
2 mg	0.85	0.61	0.38	0.30
5 mg	1.49	0.75	0.60	0.56
50 mg	2.14	1.00	0.73	0.73
Generation Average	1.49	0.79	0.57	0.53

Table 1. Average radius of concentrations tested within generations of the study.

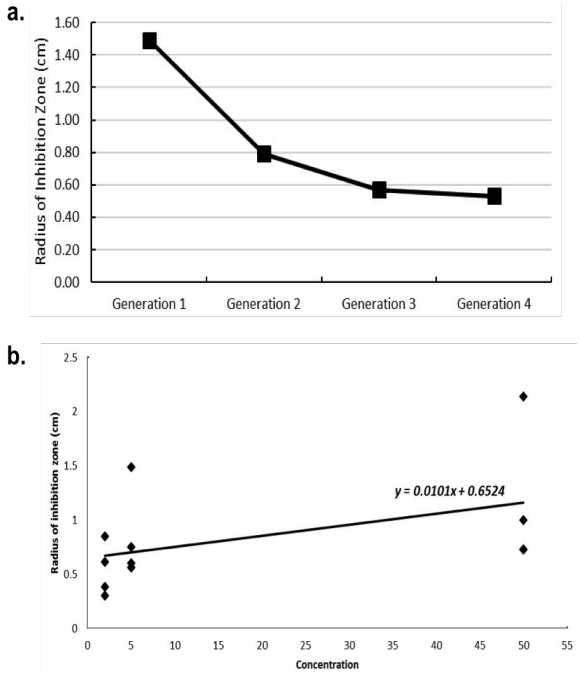

Figure 2: **Inhibition zone area.** (a) Reduction in inhibition zone area across generations, shown as radius from disc center. Increased exposure to amoxicillin throughout the four generations gave rise to antibiotic resistance. Bacteria gained most of their observed resistance upon first exposure to the antibiotic in generation 1 to generation 2. (b) Average radius of inhibition zones around discs. Points shown per concentration are for the four generations studied.

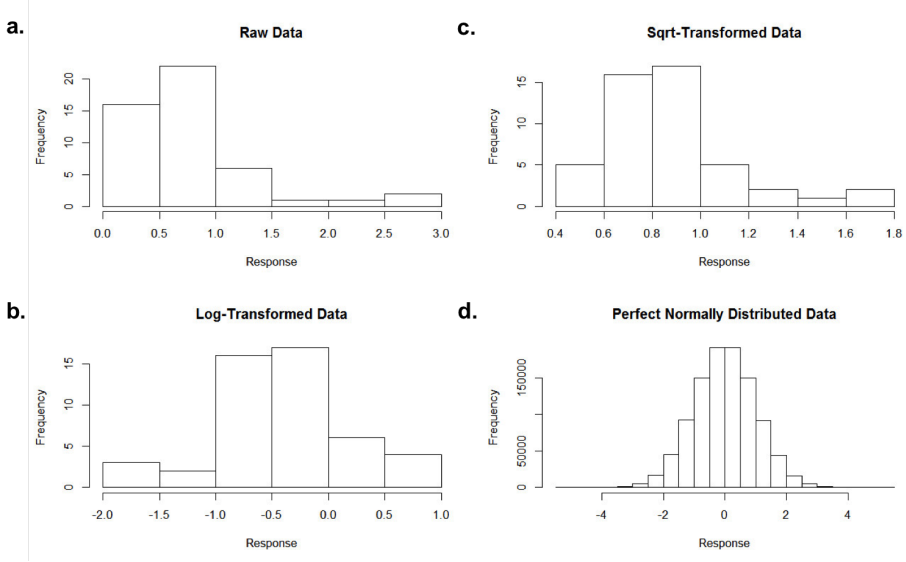

Figure 3: Histograms of radius data. (a) Histogram of the original radius data (Raw) as compared to histograms of data transformed by taking either (b) the natural log (Log-Transformed) or (c) the square root (Sqrt-Transformed). (d) A perfectly normal data set is also included for comparison.

analysis section.

Results of the regression analysis to study the impact of generation and concentration on the area of inhibition based on model [1] were summarized. For our current data, log transformation created a data set that was more normal on a histogram than that of the square root transformation (**Figure 3**). A histogram of a perfectly normal data set is also shown for comparison.

Table 2 displays results of fitting the log-transformed radius against linear and quadratic components of generation. Both components were statistically significant. The linear estimate of generation was negative, indicating a decreasing inhibition zone with repeated exposure. However, the positive quadratic estimate showed a slowing down in the shrinkage of inhibition zones as generation number increased. In R, lack of stars would be associated with effects that are non-significant; one star indicates that the effect is significant at the 5% level, two stars indicate very significant effects at the 1% level, and three stars indicate highly significant effects at the 0.1% level. The level of significance, or the *p*-value in **Table 2**, is the chance at which repeating the experiment may give different results. For example, the 2 stars of the linear effect of generation in **Table 2** indicate that upon repeating the experiment one thousand times, it is expected that different conclusions may be drawn 3.37 times (9).

	Estimate	SE	*p*-value	Significance
Generation Alone				
(Intercept)	1.166	0.350	0.00172	**
Generation - Linear	-0.988	0.319	0.00337	**
Generation - Quadratic	0.128	0.063	0.04720	*
Concentration Alone				
(Intercept)	-1.125	0.240	2.53e-05	***
Concentration - Linear	0.187	0.069	0.00947	**
Concentration - Quadratic	-0.003	0.001	0.01457	*
Both Generation and Concentration				
(Intercept)	0.383	0.266	0.15672	–
Generation - Linear	-0.988	0.212	3.03E-05	***
Generation - Quadratic	0.128	0.042	0.00367	**
Concentration - Linear	0.187	0.039	2.19E-05	***
Concentration - Quadratic	-0.003	0.001	5.71E-05	***

Table 2. Impact of generation and concentration on the radius of inhibition zone.

Next, the impact of concentration on the radius of the inhibition zone was evaluated statistically. Both the linear and quadratic components of concentration were found to be significant (**Table 2**). The *p*-values were much less than 5%, indicating that the two components significantly influenced the inhibition zone. The linear component was positive, indicating that the inhibition zone increased with lower concentrations, and the quadratic component was negative, indicating that the relationship was not linear along concentration.

When all components were fit together in model [3], they were highly significant (**Table 2**), affirming the conclusions of the current study, that both exposure to and concentration of amoxicillin had significant impact on the antibiotic resistance of *E. coli*. The significant quadratic component of generation indicates that early exposure was more important to resistance than late exposure. The significant quadratic concentration indicates that changing lower concentration levels had greater impact on resistance than changing extreme concentration levels.

The normal probability plot of **Figure 4** shows that the residuals obtained based on model [3] were normally distributed (13, 14).

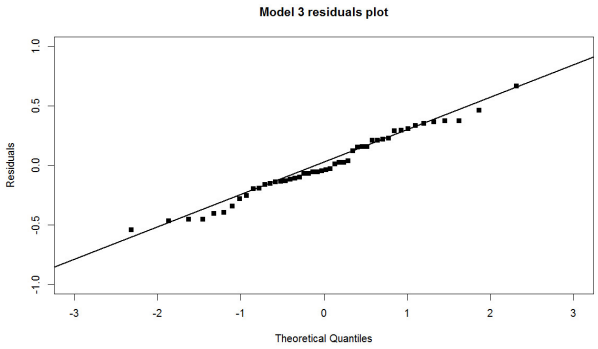

Figure 4: Model 3 residuals plot. Probability plot of residuals obtained after adjusting for the linear and quadratic components of generation and concentration. The plot shows residuals to be normally distributed.

Discussion

Exposing *E. coli* to amoxicillin and allowing them to survive and proliferate to the next cycle of growth resulted in the majority of the antibiotic resistance that was observed in the current study. The quadratic relationship between inhibition zone areas and generation number suggests that bacteria gain major resistance upon surviving early exposure to antibiotics. This experiment strongly suggests that antibiotics with optimal concentrations should be prescribed to completely clear the body of bacterial infection upon first exposure. Many recent studies reported that sublethal concentrations of antibiotics and low-level drug exposure accelerate the process of antibiotic resistance (10).

This study shows that exposing bacteria to extremely heavy antibiotic concentrations results in greater inhibition zones. However, at higher concentration levels, a 1-unit increase in concentration had a smaller impact on the radius of the inhibition zone than the same change in concentration at lower levels. The plateauing impact of concentration suggests that the optimal dosage sufficient to clear bacterial infections should be prescribed to patients without viewing concentrations heavier than optimal as a solution to antibiotic resistance, given that other factors are kept constant.

Other treatment methods for bacterial infections are needed because bacteria can be resistant to newly invented or synthesized antibiotics: exposure is a factor that promotes resistance via adaptation, but bacteria may also gain resistance without exposure. According to Spellberg (11), previously isolated bacteria were resistant to synthetic antibiotics that did not exist until the 20th century, giving evidence that bacteria could be resistant to antibiotics without exposure. As the problem of antibiotic-resistant bacteria continues to grow, the World Health Organization (12) is calling for new methods to combat bacteria, such as using phage therapy. In addition, vaccines can be used to impede bacterial infections. However, until new methods are discovered, the clinical use of antibiotics needs to be optimized.

For future potential experiments, it would be interesting to test the resistance of other species of bacteria against different types of antibiotics. Would we get similar results to the results of this experiment when different bacteria are combated with a high concentration of different types of antibiotics? The mechanisms of resistance of certain species of bacteria could also be studied in future research. Further, comparing the genetic structure of a resistant culture of bacteria against that of the non-resistant strain should help uncover novel ways to combat antibiotic resistance. Finally, instead of using Petri dishes and cultures of bacteria in a tube as this experiment has, a more relevant approach to human health could utilize data from hospitals about patient antibiotic use and dosage.

Methods

According to Michigan State University (8), there are many methods for testing antibiotic resistance including the dilution methods, the disk-diffusion method, E-Test, automated susceptibility systems, mechanism-specific tests such as the beta-lactamase detection test, and genotypic methods such as PCR and DNA hybridization methods. In this study, we used the disk diffusion method because of its simplicity, efficiency, and low cost relative to most other testing methods. In this method, commercially prepared antibiotic discs are placed over inoculated agar. However, in this study we did not use commercially prepared discs; instead, we employed custom disks with a much wider range of concentrations than are commercially available.

Serial Dilution

To obtain three different concentrations of amoxicillin, the process of serial dilution was used. Amoxicillin powder was dissolved in sterilized water several times until the desired concentrations were achieved. In this experiment, a capsule containing 500 mg of amoxicillin acted as the stock and was dissolved in 10 mL of pure water, making an antibiotic solution of 50 mg/mL. Next, 1 mL of this solution was mixed with 9 mL of pure water in another tube, diluting the concentration to 5 mg/mL. Finally, to obtain a concentration of 2 mg/mL, 4 mL from the 5 mg/mL solution was mixed with 6 mL of pure water.

Experimental Design

The three concentrations obtained through serial dilution were used to prepare antibiotic discs. The discs were made of filter paper due to its absorbent qualities. The filter paper was hole-punched, and the circular pieces from the hole puncher (0.2 cm radius) were

immersed into amoxicillin solution, where different discs were immersed in one of the three concentrations. These antibiotic discs were placed over inoculated nutrient agar to prevent bacterial growth in the area near the disc. The area in which bacteria cease to grow around antibiotic-saturated discs is known as the inhibition zone. The smaller the inhibition zone, the more resistant bacteria have become.

In each generation, 4 discs were immersed into one of 3 concentrations and placed in Petri dishes. To achieve a standardized and efficient experiment, a novel experimental design was created and employed (**Figure 1a**). In each generation, 2 Petri dishes were used. In each Petri dish, there were four quarters. The quarters were labeled 0, 1, 2, and 3 for control, 2 mg/mL, 5 mg/mL, and 50 mg/mL, respectively. The control quarter was left without any antibiotic discs to monitor regular bacterial growth. In each of the other quarters, there were 2 sections in which antibiotic discs with the same concentration were placed (**Figure 1a**). Petri dishes were divided and labeled with marker on the bottom outside of the agar dish.

Procedure

A tube of pure *E. coli* broth was obtained from Carolina Biological Supply Company, Burlington, NC. *E. coli* broth was cultured in the first generation by inoculating the nutrient agar in 2 Petri dishes, divided as explained earlier. Then, the antibiotic discs were placed in the sections that corresponded to their concentrations. The two Petri dishes were then placed in an incubator upside down for 48 hours at 37°C. Radii of inhibition zones were measured right after the incubation period.

In the second generation, the surviving *E. coli* bordering the inhibition zones from generation 1 were cultured onto 2 new Petri dishes in corresponding quarters. For example, the surviving *E. coli* from quarter 1 were used to inoculate quarter 1 of generation 2 Petri dishes. Cultures bordering the inhibition zone were used rather than cultures growing farther away, as bacteria that grew nearer the inhibition zones were believed to be more resistant. Antibiotic discs were then placed in corresponding sections as explained for generation 1. Similar to those of generation 1, the Petri dishes of generation 2 were placed in an incubator for 48 hours at 37°C and the inhibition zones were measured after incubation. The same procedures of inoculation, disc placement, incubation, and measurement were followed for generations 3 and 4. After the experiment, all Petri dishes with *E. coli* culture were bleached for an hour before they were disposed of.

Statistical Analysis

The impact of generation and concentration on the area of inhibition was studied by regression analysis. R statistical software (9) was used to fit the radius against linear and quadratic components of generation. The quadratic component was simply the square of generation number and it explains nonlinear changes in radius per generation. As **Figure 2a** shows, the change in radius was greater in early than in late generations, which was tested statistically by fitting a quadratic component in the analysis. The model of analysis is described in [1], which is a second-degree equation:

$$\text{Log}(Y) = \mu + \alpha G + \beta G^2 + \varepsilon \qquad [1]$$

where Y represents the radius, μ symbolizes the intercept, α and β are the regression coefficients, G is the generation number, and ε represents random unexplainable effects. Radius data were log-transformed to normalize the dependent variable, Y (13, 14).

Similar to the procedures followed with generation, the impact of concentration on the radius of the inhibition zone was evaluated statistically. The model of analysis is described in the following second-degree equation [2],

$$\text{Log}(Y) = \mu + \alpha C + \beta C^2 + \varepsilon \qquad [2].$$

where μ symbolizes the intercept, α and β are regression coefficients of radius, Y, C represents concentration, and ε represents random unexplainable effects.

Finally, all components, linear and quadratic components of generation and concentration, were fit together using the following regression model.

$$\text{Log}(Y) = \mu + \alpha_1 G + \beta_1 G^2 + \alpha_2 C + \beta_2 C^2 + \varepsilon \qquad [3].$$

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