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Article

Effects of Prolonged Azithromycin Therapy on Bacterial Resistance to Functionally Analogous Antibiotics

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SUMMARY

Bacteria may be innately resistant to antibiotics or acquire antimicrobial resistance through a variety of mediums. Under certain conditions, bacteria develop cross-resistance, a reduction in susceptibility to many antibiotics they have never been exposed to before. Most frequently, chemical similarities between antibiotics cause cross-resistance since bacterial defenses are counteractive to a specific molecule. However, cross-resistance to many chemically different antibiotics can occur when bacteria mutate to develop non-specific defenses. This study investigated a subject who had received prolonged azithromycin therapy for a neuropsychiatric condition related to chronic Group A Streptococcus infection. Given the possibility of cross-resistance, we hypothesized that, after prolonged azithromycin therapy, any bacteria collected from the subject would be resistant to structurally analogous antibiotics. We also hypothesized that, if bacteria from the subject had developed metabolic mutations, resistance to functionally analogous antibiotics would be present. From a series of antibiotic susceptibility tests, we concluded that the subject bacteria were resistant to erythromycin, a structural analog of azithromycin, but exhibited standard sensitivity to functional antibiotic analogs. The results of our study will help identify the risks associated with prolonged antibiotic therapy for a variety of conditions.

INTRODUCTION

The positive effects of antibiotics on human health have been unparalleled by any other pharmaceutical for the past five decades. Within recent years, however, bacterial resistance to antibiotics has grown exponentially as new resistant mutants continue to develop and spread (1). The World Health Organization (WHO) states that excessive prescription of antibiotics is a primary contributor to the development of resistance, which can occur for many reasons, such as over-the-counter availability (2). Our ability to control the spread of human pathogens has slowed as the rate that bacteria gain resistance far exceeds the rate at which we create new antibiotics. Additionally, studies have shown that the use of antibiotics limits the ability of human systems to perform vital functions and shield against future infections (3, 4). Because of these factors, many common infections associated with antibiotic therapy are becoming more difficult, if not impossible, to treat, making antibiotic resistance one of the biggest threats to universal health (2).

Bacteria may have innate resistance or gain resistance to one or more classes of antibiotics through genetic mutations and gene transmission from one bacterium to another. Bacteria survive by employing these resistance mechanisms to counteract the effect of the antimicrobial agents (5). In the context of this study, antibiotics that function by inhibiting protein synthesis are rendered less effective when bacteria change their ribosome structure (6). Modifications, such as this, are how bacteria develop cross-resistance, a reduction in susceptibility to many antibiotics they have never been exposed to before (7). Szybalski and Bryson (1952) reported that chemical similarities between antibiotics are most often the cause of cross-resistance because bacterial defenses are counteractive to a specific molecule (8). However, cross-resistance to many chemically different antibiotics can also occur when the metabolic pathway they attack is altered (8). For example, macrolide antibiotics rely on inhibiting the translation of mRNA into protein. If bacteria alter their ribosomes in response, the efficacy of all functionally similar antibiotics decreases. Following these findings, a study by Gutmann and colleagues (1988) found that mutations correlated with cellular metabolic activity, such as reduced membrane permeability, can cause resistance to multiple antibiotic classes as well (9).

In this case study, we studied the effect of prolonged antibiotics on bacterial resistance development on a subject who had received azithromycin therapy for over five years to treat a pediatric autoimmune neuropsychiatric disorder associated with streptococcus (PANDAS) (10). PANDAS is a disorder characterized by the development of obsessive-compulsive disorder (OCD), motor tics, and abnormalities in behavior. The disease traditionally manifests in younger patients and is associated with the presence of Group A streptococcus (GAS) infections. Prolonged use of antibiotics (most commonly beta-lactams and macrolides) to prevent future streptococcal infections is appropriate for severe cases of this disorder (10). Currently, we are limited in our understanding of the effects of extensive azithromycin therapy on bacterial resistance in patients. Affected bacteria may have directly evolved mutations to counteract the mechanisms of antibiotics or received resistance genes from other bacteria.

Considering the possibility of cross-resistance, the

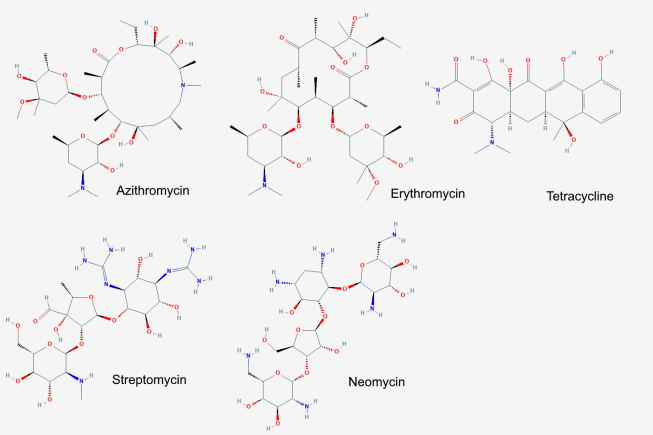

Figure 1. Molecular structures of azithromycin, erythromycin, tetracycline, streptomycin, and neomycin. Top row, from left to right: azithromycin, erythromycin, tetracycline. Bottom row, from left to right: streptomycin, neomycin. Note the similarities between macrolides erythromycin and azithromycin. Also, note the similarities between aminoglycosides neomycin and streptomycin. Depictions of molecular structures obtained from PubChem.

aim of this work was to elucidate the effects of prolonged azithromycin therapy on bacterial resistance in the subject. Erythromycin, tetracycline, streptomycin, and neomycin were selected because they are functionally analogous to azithromycin. These antibiotics have similar ways of inhibiting bacterial growth; however, they do not all have similar chemical structures (11-15). Of the four antibiotics, erythromycin is the only one structurally analogous to azithromycin, while tetracycline, streptomycin, and neomycin have dissimilar structures (**Figure 1**). We hypothesized that after prolonged azithromycin therapy, any bacteria collected from the subject would be resistant to structurally analogous antibiotics. Additionally, under the condition that bacteria from the subject had developed mutations, we predicted that resistance to antibiotics that are functionally analogous to azithromycin would be present.

Our results indicated that the bacteria collected from the subject were completely resistant to erythromycin while having above standard sensitivity to tetracycline, streptomycin, and neomycin. Overall, the results of this case study will aid in identifying the risks associated with antibiotic treatment significantly longer than the standard duration.

Trial #	Erythromycin (mm)	Tetracycline (mm)	Streptomycin (mm)	Neomycin (mm)	Control (mm)
1	0	40	20	32	0
2	0	40	22	36	0
3	0	38	18	34	0
4	0	42	18	32	0
5	0	38	17	28	0
Average	0	39.6	19	32.4	0
SD	0	1.67	1.79	2.97	0

Table 1. Raw data for each of the five trials. The diameters (mm) of the inhibition zones after 18 hours of incubation were recorded. Four different antibiotics disks were tested along with one blank disk for a control. The average diameters for each disk and the standard deviation across all trials were also calculated.

RESULTS

Rationale for Interpretation

To interpret our results, we compared the recorded data (**Table 1**) to interpretive standards for antibiotic susceptibility (**Table 2**). Health professionals create interpretive standards to indicate the sensitivity of bacteria to an antibiotic based on the size of their zone diameter. We obtained values for the interpretive standards of each antibiotic from a study by Sarker *et al.* (16) which used the disk diffusion method and swabbed bacteria from similar locations. There are different acceptable diameters for each antibiotic depending on the concentration used, however, **Table 2** corresponds with the standardized concentrations determined to be most effective. The antibiotic disks we used in this study were of the standard concentrations of erythromycin (15 µg/disk), tetracycline (30 µg/disk), streptomycin (10 µg/disk), and neomycin (30 µg/disk). By comparing the values recorded in **Table 1** to the standards presented in **Table 2**, we were able to interpret the ability of each antibiotic to inhibit the growth of the subject bacteria. For measurements that fell within the range of the "sensitive" column for the respective antibiotic, we determined that the bacteria responded normally. Additionally, we assumed that a normal response indicated that there were no significant changes in resistance as a result of the treatment. For measurements that fell within the range of values in the "resistant" column, we concluded that the subject bacteria developed resistance mechanisms to that antibiotic at the effective concentrations. No measurements in the present study fell within the "moderately sensitive" range.

Resistance to Erythromycin

We exposed oral bacteria collected from the subject to erythromycin, neomycin, streptomycin, and tetracycline. After each trial, we assessed the bacterial growth on the agar plates visually. We assumed that, if the bacteria tested were sensitive to an antibiotic disk, they would not grow around it. In contrast, if the bacteria were resistant to the antibiotic, they would grow closer to the disk. We also evaluated the difference in bacterial growth by measuring the diameter of

Name of Antibiotic (Dose)	Sensitive (mm)	Moderately Sensitive (mm)	Resistant (mm)
Erythromycin (15 µg/disk)	≥23	14-22	≤13
Tetracycline (30 µg/disk)	≥15	12-14	≤11
Streptomycin (10 µg/disk)	≥15	12-14	≤11
Neomycin (30 µg/disk)	≥17	13-16	≤12

Table 2. Zone diameter (mm) interpretive standards for the determination of antibiotic sensitivity and resistance status. Values were obtained from Ref. 16. Susceptibility can be approximated by comparing raw data in Table 1 to the ranges in the table.

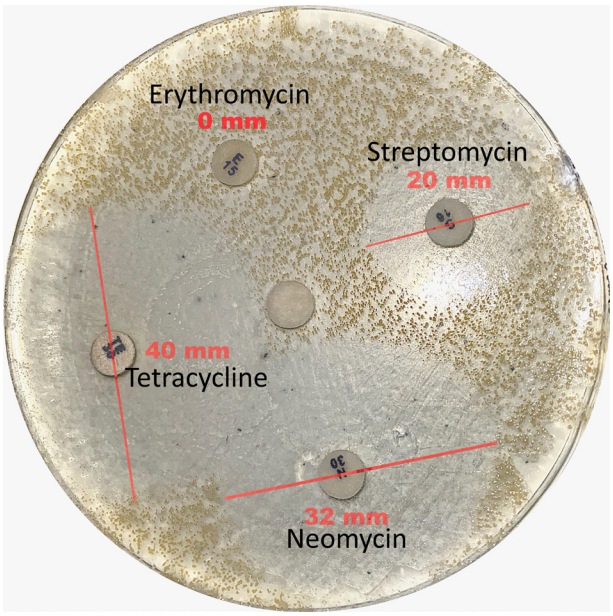

Figure 2. Image of the bacterial growth after trial one. Clockwise: (0 mm) inhibitory zone for erythromycin, (20 mm) inhibitory zone for streptomycin, (32 mm) inhibitory zone for neomycin, (40 mm) inhibitory zone for tetracycline. Note the variation in growth between the blank control and the antibiotic diffusion disks.

the circular zone where no bacteria had grown. If the zone expanded beyond the boundaries of the plate, we doubled the distance between the center of the disk to the nearest colony. Colonies of bacteria appeared to grow consistently outside of a certain range of the antibiotic disks, excluding the control and erythromycin, which had no visual signs of growth inhibition (**Figure 2**). After recording these observations over five trials, we concluded that the subject bacteria had developed resistance to erythromycin.

Susceptibility to Functional Analogs

The raw data for the five subject trials (**Table 1**) showed that on average, neomycin, streptomycin, and tetracycline exceeded the interpretive standard for sensitivity by varying amounts. We visually represented the data by calculating the average points for each disk and plotting them graphically in comparison to the standard sensitivity (**Figure 3**). We noted that the average inhibitory zones of tetracycline and neomycin were significantly higher than the interpretive standard for sensitivity. There was a less significant difference between the average diameter and the standard for streptomycin. Based on these results, we concluded that the subject bacteria were susceptible to the other three antibiotics. Additionally, we calculated the average fold-change to determine the difference between the interpretive standards and the experimental values for each antibiotic. As seen in **Figure 4**, there was a significant difference in zone diameter from the standard for sensitivity in neomycin and tetracycline. We consider the validity of this result in more detail in the Discussion section.

DISCUSSION

In this study, we investigated a subject who has been receiving prolonged azithromycin antibiotic therapy for a neuropsychiatric condition related to chronic GAS infections (10). We had one major finding based on the data collected, as well as inferences that could be supported by further experiments. We concluded that the subject bacteria were completely resistant to erythromycin, an antibiotic belonging to the same class as the azithromycin used in therapy. This resistance is most likely a result of chemical-specific defensive mechanisms since azithromycin and erythromycin are structural analogs with only a few variations in their molecular compositions. As seen in **Figure 1**, the majority of the structure remains the same, as azithromycin is a derivative of erythromycin (15). We hypothesized that the differences in these molecules may affect how they distribute throughout the body and into cells; however, our study has shown they do not affect the mechanism of inhibition.

Tetracycline, streptomycin, and neomycin are considered functional analogs to azithromycin. These antibiotics inhibit bacterial growth in similar ways by affecting mRNA translation (12-14), but their molecular compositions vary quite significantly (**Figure 1**). If our conditional hypothesis was correct, resistance to these three other antibiotics would indicate the presence of bacteria that have mutated nonspecific defense mechanisms. However, this was not the case and suggests that the previously mentioned mutations were absent. We speculated that the mechanisms found in the tested bacteria were specific to the location the antibiotics bind to, as both erythromycin and azithromycin bind to the 50S subunit of bacterial ribosomes (11, 15). In the case that these nonspecific mutations were present, we expected that they would affect processes such as material intake through the cell wall or the pathway in which proteins are assembled. This would limit the access of antibiotics to parts of the cells that must be shut down to stop bacterial growth. In a future study, we could determine the correlation between metabolic changes and susceptibility by evaluating these two factors separately.

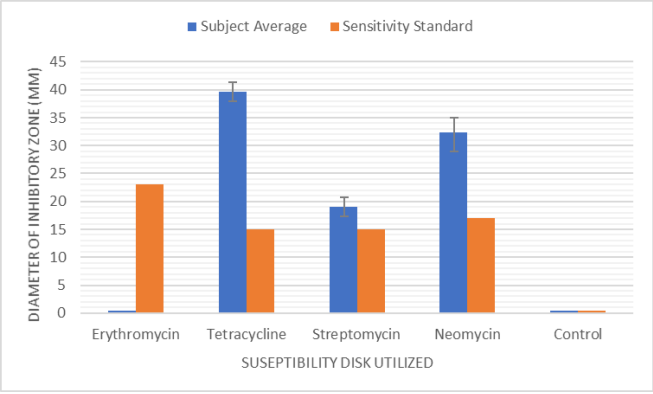

Figure 3. Comparison of subject average zone diameters (blue) with the interpretive standard for sensitivity (orange). Bacterial growth inhibition occurred for every disk except erythromycin and the control. Error bars represent standard deviation.

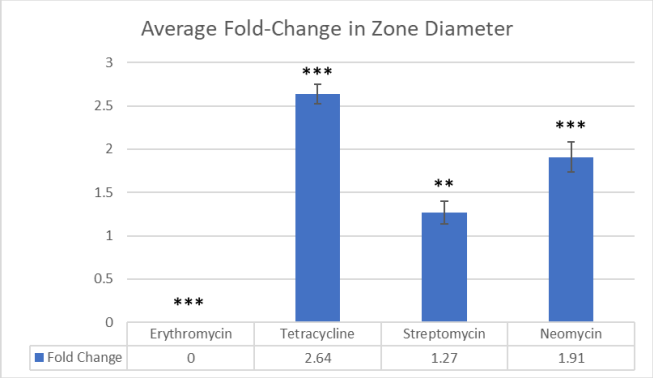

Figure 4. Average fold-change in diameter relative to the minimum sensitivity standard for each antibiotic. Values greater than one indicate an increase, while values less than one indicate a decrease. Error bars represent standard deviation. Asterisks indicate significance of the changes (**p-value<0.01, ***p-value<0.001).

Though zone diameter can vary above the standard depending on the subject, we questioned the significance of the results in **Figure 4**. The basis behind another treatment method, combination antibiotic therapy, is that resistance to one antibiotic may increase susceptibility to another (17). The chances of resistance to one or more antibiotics utilized are significantly lower compared to the use of a single antibiotic (17). Although we speculated that the susceptibility of the subject's sampled bacteria could be influenced by the factors making combination therapy successful, we did not identify the specific strain or mixture of strains we isolated. In future studies, we could accomplish this by observation of the bacteria under a microscope, or by Gram staining to classify.

We determined the concentration of any infection before the procedure by measurement through blood work. The subject patient had recently been tested and confirmed to have insignificant levels of the pathogen in their system, suggesting the absence of erythromycin-resistant streptococcus. Based on the health of the subject at the time of the study, it is unlikely there were any other pathogenic strains to interfere with the results obtained. Though the presence of foreign, non-pathogenic, erythromycin-resistant strains was a possibility, we concluded that while not a result of selective evolution in the subject, this could still act as an expression of the risk of prolonged treatment.

There were two sources of error in our experimental process. Firstly, we lacked control over air contaminants due to the inaccessibility of lab equipment during the time we conducted the study. We followed sterilization protocol rigorously during the preparation of the plates and before incubation using isopropyl alcohol and flame sterilization of tools used to minimize the risk of contamination. We attempted to make this source of error more negligible by testing environmental bacteria in the proximity of where we completed the procedure. The samples we collected proved to be sensitive to the antibiotics, implying that the only bacteria within the resistant ranges could be from the subject. Secondly, the bias in the collection of raw data using a ruler was a source of error during the procedure; however, we completed multiple trials and calculated the standard deviation between results calculated to make this more negligible.

There are also revisions to our experimental process that would improve the viability of the results we obtained. Although we based the experiment on the assumption that the subject bacteria were resistant to azithromycin, the addition of an azithromycin disk to the susceptibility test could confirm or deny any speculations about erythromycin-resistant strains. The procedure also lacked the presence of a non-resistant control to compare results with. Although the interpretive standards act as a reliable baseline, we were not able to obtain precise results with the procedure here. In future studies, the presence of a control subject could solidify our findings.

Lastly, characterizing the effect of prolonged exposure to antibiotics on specific species of bacteria could have a wider-reaching impact in this field. In the context of PANDAS, many patients would benefit from research on specifically Group A streptococcus infection and its response to prolonged antibiotic exposure. Although we considered the changes expressed in **Figure 4** statistically significant, they lack meaning in the context of this study as the bacteria tested were not identified. We could address the biological significance in a future study, where the development of resistance may vary depending on the species and their properties.

The results from this study are meaningful in the context of illnesses associated with chronic bacterial infections, such as PANDAS, a recurring infection, and Mycobacterium, which are highly difficult to kill. Treatment options for these conditions demand extended durations of many months to even years. With knowledge of the rapid spread and dangers associated with microbial resistance mechanisms, prospective patients may be hesitant to undergo such therapy. The results of this case study should aid prospective patients in identifying the risks associated with antibiotic treatment significantly longer than the standard duration.

MATERIALS AND METHODS

Collecting the Samples

Bacterial samples were collected from the teeth, gums, and throat of the subject using a sterile swab, then directly streaked onto a plate containing nutrient agar and incubated at 35°C for 24 hours. After colonies had formed, a single colony was isolated with an inoculating loop and transferred to a tube containing 10 mL of LB liquid broth medium (American Bio Innovations). The tube was incubated at 35°C for 18 hours, shaken regularly until turbidity was visible. SRC/IRB approval for human subject research was not required since the contributor of the samples was the author.

Preparing the Plates

The bacteria were collected from the tube by dipping a sterile swab into the medium, then rotating it against the side

to remove excess fluid. The bacteria were streaked onto five different plates containing 15 mL of Mueller Hinton nutrient agar (Carolina Biological). To ensure even distribution, bacteria were swabbed three times over the entire agar surface, rotating the plate approximately 60° each time. For the details of these methods, the Kirby-Bauer disk diffusion susceptibility test protocol was referenced (18).

Applying the Antibiotic Disks

Antibiotic susceptibility testing disks (Carolina Biological) were kept frozen at -18°C in a desiccated container until the day of use to ensure the validity of the results. The antibiotic disks were set out to equilibrate with room temperature for two hours before the procedure began. Plates were divided into four different sections, one for each antibiotic and a blank control disk in the center (Carolina Biological). The disks were placed in the center of their sections and pressed down lightly to ensure contact with the agar. The plates were then flipped upside down and incubated at 35°C for 18 hours. Before the study, a plate with bacteria collected from the environment was incubated to verify the efficacy of each antibiotic disk.

Measuring Inhibitory Zones

The diameter of the inhibition zones was measured to the nearest millimeter using a ruler. If the edge of the inhibitory zone fell beyond the edge of the plate and could not be measured, the distance from the center of the antibiotic disk to the edge of the zone was taken and multiplied by two to find the diameter.

Statistical Analysis

The susceptibility tests were repeated for a total of five trials. The standard deviations and means of the collected data were calculated for each type of antibiotic disk used based on the data presented in **Table 1**.

For **Figure 4**, the fold-changes for five trials of each antibiotic were calculated and then averaged. To determine the fold-change of each trial, the formula B/A was used, where B was the measurement from that trial, and A was the interpretive standard minimum for sensitivity. We also used an unpaired t-test to determine the *p*-values for each comparison. Due to the polarity of our results, performing additional tests was unlikely to change our conclusions.

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