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Amanda Khoury

The potential anti-microbial use of Azithromycin against multi-drug resistant *Burkholderia cepacia* complex in Cystic Fibrosis patients

Abstract:

Cystic Fibrosis (CF) is an autosomal recessive disease that affects thousands of individuals worldwide. The disease is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene resulting in the dysregulation of apical ion channels. This disruption in ion transport leads to the production of the characteristic thick mucus seen in patients with CF. Although many organ systems are affected, the hallmark of the disease is viscous mucous plugs in the respiratory tracts leading to the disruption of mucociliary clearance and chronic bacterial colonization of the airways.[1–3] Infections by organisms of the *Burkholderia cepacia* complex (BCC), a group of about 17 closely related species of Gram-negative bacteria, are associated with fulminant necrotizing pneumonia and severe decline in CF lung function.[4,5] Acute exacerbations of CF are treated rapidly with antibiotics to halt further destruction of the lung. This repeating antibiotic exposure, however, has naturally led to the emergence of multidrug-resistant (MDR) organisms. Azithromycin, a macrolide antibiotic with anti-inflammatory properties is considered one of the standard-of-care therapies for patients colonized with *Pseudomonas aeruginosa*, and is commonly administered three times a week. Azithromycin treatment has been shown to decrease the frequency of exacerbations, and has largely been attributed to azithromycin's anti-inflammatory properties[6], since the drug does not show direct bacterial killing of the pathogen in standard minimum inhibitory concentration (MIC) tests in bacteriologic media.[6] However, recent data from Lin and Nizet et al[7] suggests that azithromycin may indeed have direct antimicrobial effects on *P. aeruginosa* and other Gram-negative bacteria, which are appreciated only under more physiological conditions (e.g. tissue culture media) and in synergy with cationic antimicrobial peptides of the host innate immune system.[7,8] However, the effectiveness of azithromycin in patients with BCC is largely unknown. In this project, we investigate the effects of azithromycin against BCC in CF patients, with special attention to functional interactions with host defense factors. This research can lead to a better understanding of the future use of Azithromycin to reduce the development of MDR infections as well as its use as an anti-microbial agent against these infections.

Introduction:

Cystic fibrosis (CF) is the most common inherited disorder of Caucasian populations with over 70,000 individuals affected worldwide. It is an autosomal recessive disease that is caused by a mutation in the CFTR (cystic fibrosis transmembrane conductance regulator) gene. Over 1600 different mutations of the CFTR gene have been described, however the most common mutation is the deletion of phenylalanine at codon 508, which is found in 70% of patients with CF. The CFTR gene encodes an apical ion channel that regulates transport of chloride and bicarbonate in epithelial tissues of the airways, pancreatic ducts, sweat ducts, intestines, biliary tree and vas deferens. The dysregulation of the ion transport leads to thick, viscous secretions that lead to inflammation, tissue damage and destruction affecting multiple organ systems.[1,9]

Although multiple organ systems are affected, for patients with CF, obstructive lung disease is the primary cause of morbidity and is responsible for 80% of mortality. In the lungs, the thick mucus leads to mucous plugs and failure of mucociliary clearance of bacteria in the lungs, which is the lung's innate defense mechanism. The lungs are therefore not able to effectively clear inhaled bacteria. In addition, there is an excessive inflammatory response to pathogens. Eventually the airway becomes colonized with bacteria leading to a vicious cycle of infection and inflammation and finally airway remodeling and destruction.[1,2]

There are many bacterial pathogens that are commonly found in the airways of CF patients including *Staphylococcus aureus, Pseudomonas aeruginosa* and Gram-negative rod (GNR) organisms such as *Strenotrophomonas matophilia* and *Burkholderia cepacia.* Infective exacerbations in patients with CF are treated promptly to minimize accelerated damage to lungs. However, due to greater patient longevity and increased antibiotic use, physicians are increasingly being faced with infection with multi-drug resistant isolates.[6]

One of the most threatening pathogens in CF is *Burkholderia cenocepacia,* a member of a group of about 17 species that make up the *Burkholderia cepacia complex. B. cenocepacia* is an opportunistic pathogen that has been shown to lead to severe decline in CF lung function possibly developing into a systemic infection known as cepacia syndrome.[4,10] *B. cenocepacia and B. multivorans* predominate in CF and account for approximately 85-97% of all BCC infection in CF.[10] According to the data from the CF Foundation Patient registry, only 3% of patients with CF had a positive culture for BCC, however it is among the most virulent; and chronic infection has been associated with increased morbidity and mortality.[11] *B. cenocepacia* is naturally multi-drug resistant to different classes of antibiotics and its pathogenicity is promoted by several virulence determinants making treatment extremely challenging.[5] Therefore, it is vital to investigate and identify innovative therapeutic approaches to decrease the selective pressure leading to drug resistant organisms as well as new treatment options once they have developed.

Azithromycin, a macrolide antibiotic has been widely used as chronic therapy for CF patients with significant reduction of pulmonary exacerbations. The current guidelines recommend that patients 6 years or older who are chronically colonized with *P. aeruginosa* receive azithromycin 3 times a week to reduce exacerbations and the need for additional antibiotics.[6,12] This has largely been attributed to azithromycin's intrinsic anti-inflammatory properties by decreased pro-inflammatory gene expression.[12] When studied in traditional assays in bacteriologic media, *P. aeruginosa* and other GNRs are non-susceptible to azithromycin.

However, new data from Lin and Nizet et al[7] has suggested that azithromycin may have direct antimicrobial effects *in vivo* in murine model of *P. aeruginosa*, showing the antibiotics ability to synergize with host innate immune factors such as anti-microbial peptides that can target the outer cell membrane. Additionally, data from Kumaraswamy and Nizet[8] have observed bactericidal activity of azithromycin against Gram-negative *Stenotrophomonas maltophilia* (SM) that was absent in MIC checkboard assays traditionally tested in bacteriological media. In this project we attempt to reexamine the potential of

azithromycin to combat gram-negative MDR, such as BCC under more physiological medium conditions. This project will help us understand the future utility of azithromycin in decreasing the multidrug resistant infections as well as acting as an anti-microbial agent against MDR gram-negative rods.

Materials/Methods:

Bacterial strains, media and antibiotics

B. cenocepacia AU 16621 and *B. cepacia* AU 26227 from University of Michigan *Burkholderia* biorepository isolate strains were utilized in experiments. Isolates were stored in Luria broth (LB) + 70% glycerol at -80˚C until use. The stored isolates were used to streak plates, which were then incubated for 24-48 hours until small colonies were observed. Overnight cultures were prepared using isolated colonies on the streaked plates. Azithromycin was purchased from Sigma-Aldrich and aliquoted to 12,500 µg/ml and stored at -20˚C. Bacteriological medium Muller-Hinton broth (Spectrum Chemicals) was supplemented with 10mg/ml Ca2+ and 10mg/ml µl Mg2+ in 50 ml MHB (CA-MHB). Tissue culture medium Roswell Park Memorial Institute 1640 (RPMI) (ThermoFisher Scientific) was used and various supplements including fetal bovine serum (FBS), Brain Heart Infusion (BHI), LB, and bicarbonate were assessed in order to optimize bacterial growth.

Growth Assays

Overnight cultures were grown in 5ml LB and put in 37˚C shaking incubator. Manual growth assays were performed in LB, CAMHB and RPMI with various supplements described in the results. Bacteria from the overnight culture was added to the various media and diluted to a standard starting OD of 0.2 (late stationary growth phase). Negative controls were wells with only media and no bacteria. The bacteria was then incubated in the 37˚C shaking incubator and the OD was measured at various time points.

Bioscreen growth assays were set up in a similar fashion using Honeycomb well plates. BioscreenC machine was set up to read the plates at an OD600nm every thirty minutes for 20 hours with continuous shaking.

MIC, and Bactericidal Activity

Azithromycin minimal inhibitory concentration (MIC) assays were performed in CA-MHB according to CLSI guidelines and in various tissue media conditions as described above. Azithromycin was serially diluted to produce final concentrations of 64 µg/ml to 0.25 µg/ml. An initial OD of 0.5 was diluted to a final dilution of 5E6 colony forming units of bacteria per well. Percent of bacterial survival was calculated by comparing each well to the positive control set to 100% survival. The MIC was determined to be the lowest concentration of Azithromycin required to result in less than 10% bacterial survival. Bactericidal activity of Azithromycin was assessed using Resazurin and observation of color change.

Results:

In order to compare the minimal inhibitory concentration of azithromycin in the conventional CA-MHB media versus RPMI, it was first necessary to optimize the growth of *B. cenocepacia* in RPMI. Manual and BioscreenC growth curve assays were performed varying RPMI supplementation. An overnight culture of *B. cenocepacia* was prepared in LB. For the manual screen, the bacteria were diluted to a starting OD of 0.1 (calculated to be 6.3 x 10^7 CFU/ml) using varying media: LB, CA-MHB, RPMI + 20%LB and RPMI + 20%LB + 10%FBS. The bacteria were then incubated in 5ml Falcon tubes and growth was assessed at OD 600nm at 2hrs, 4hrs, 6hrs and 24hrs. **Figure 1** shows adequate growth in all media in 5ml tubes, with optimal growth in RPMI supplemented with LB an FBS.

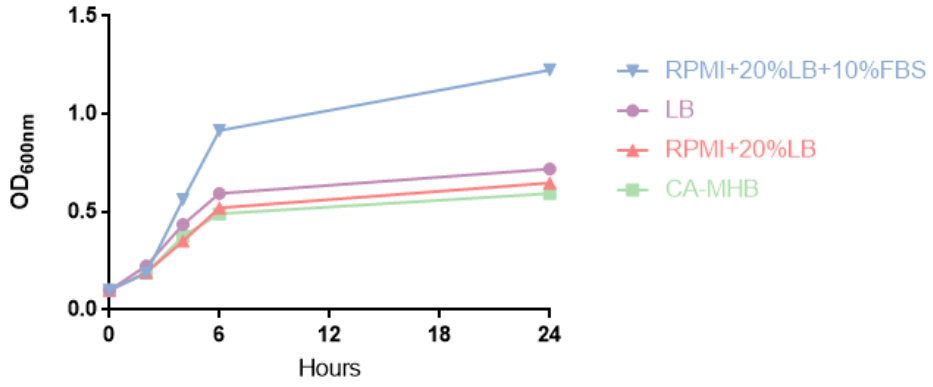


Figure 1: Manual Growth Assay of *B. cenocepacia*. Comparative growth assay performed in LB, CA-MHB and supplemented mammalian tissue culture (RPMI+1o%LB and RPMI+20%LB+10%FBS) media.

Next, we wanted to assess if we would see similar growth patterns when using small volume wells versus large volumes tubes, in order to better emulate the conditions of standard MIC testing. We performed a 24-hour growth curve of *B. cenocepacia* using the BioscreenC. An overnight culture was prepared in LB and the bacteria was diluted to a starting OD of 0.1 in LB, CA-MHB, and supplemented tissue culture media (RPMI+LB, RPMI+FBS and RPMI+LB+FBS). 200 µl of each bacteria containing media was loaded into the BioscreenC honeycomb well plate. The BioscreenC machine assessed OD of each well every 30 minutes over a 24-hour time period. The results showed adequate growth of *B. cenocepacia* in LB and CA-MHB, however no growth in RPMI was observed.

One hypothesis of the lack of growth in RPMI was that we were shocking the bacteria when changing the media from which it was grown in overnight to a different media used in the experiment. We repeated the experiment above, this time growing the overnight cultures in the corresponding media in which they would be plated in the next day. Overnight cultures

were grown in LB, CA-MHB and supplemented tissue media (RPMI + 20%LB and RPMI + 10%FBS). The bacteria were then diluted to a starting OD of 0.2 and 200 µl was loaded into the honeycomb well plate. A 24-hour growth assay was performed again using the Bioscreen C. Unfortunately, the results were similar to our initial Bioscreen growth assay, as there was adequate growth in LB and CA-MHB, but still no growth in RPMI (**Figure 2**).

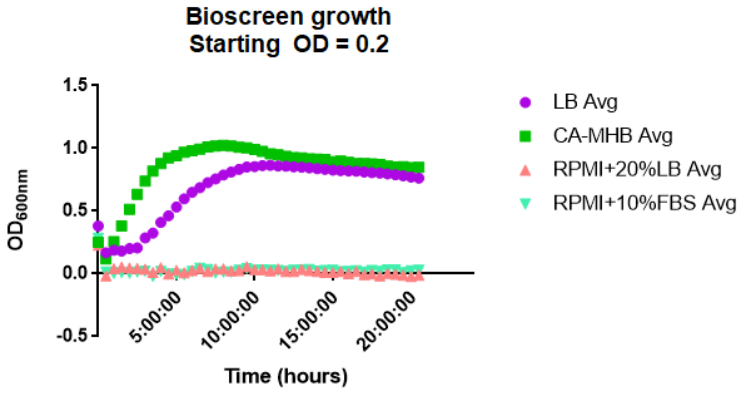
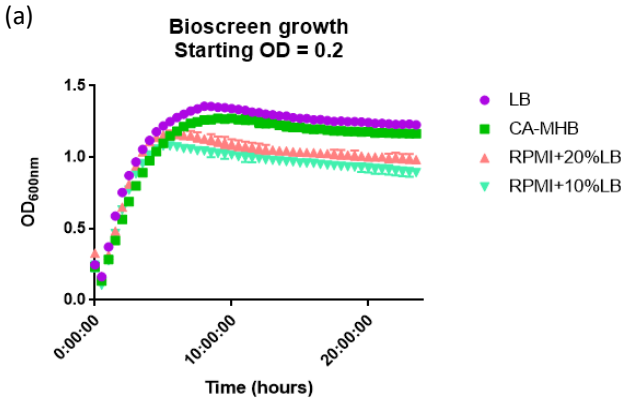
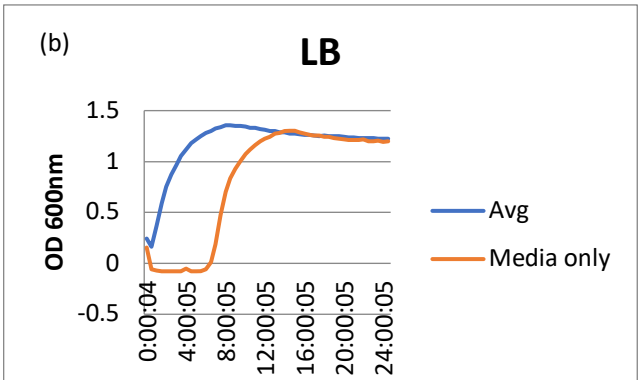
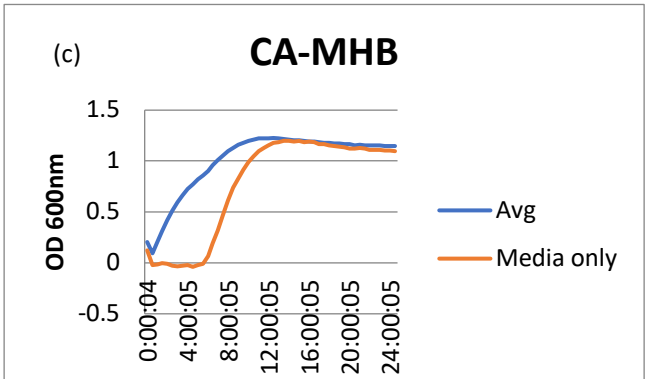


Figure 2: 24 hour BioscreenC growth assay of *B. cenocepacia.* Comparative 24-hour small well growth assay of *B. cenocepacia* in LB, CA-MHB and supplemented tissue culture media (RPMI+20%LB and RPMI+10%FBS). Bacteria was loaded into triplicates, with average optical density at each time point shown on the graph.

We repeated this experiment again, however we were especially cautious as to not remove the bacteria from the incubator until we were ready to immediately plate. A BioscreenC assay was done again in CA-MHB, LB and supplemented tissue culture media (RPMI+20%LB and RPMI+10%FBS). The results initially showed adequate growth of *B. cenocepacia* in all tested media. However, further examination showed growth in the negative, media only control, indicating likely contamination of the LB and CA-MHB medias. The RPMI supplemented with FBS showed adequate growth of *B. cenocepacia* with no growth in the negative control. This was the first time we were able to demonstrate adequate growth of BCC in tissue culture media (**Figure 3a-e)**.







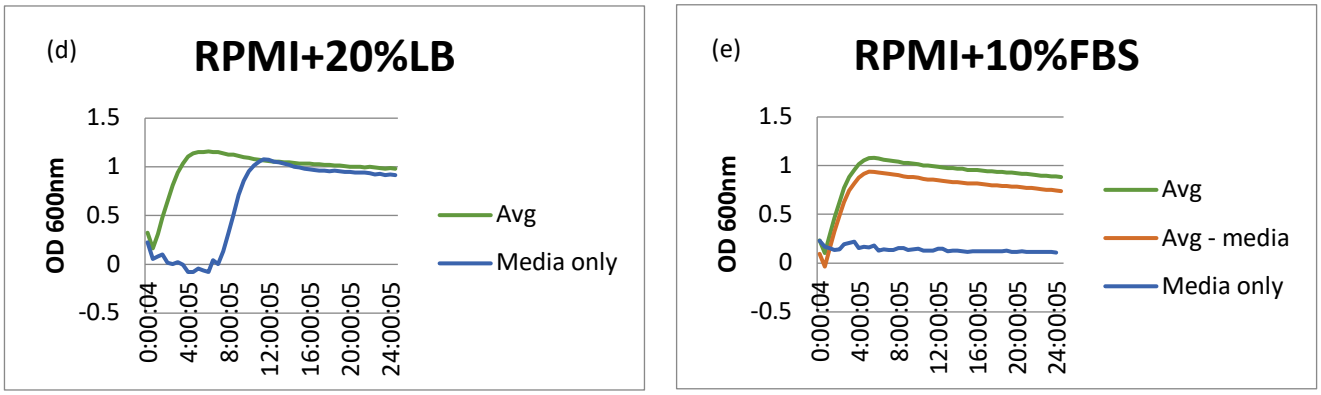


Figure 3a-e: Repeat BioscreenC growth assay of *B. cenocepacia*. (a) Average OD of bacteria containing media, LB, CA-MHB, supplemented tissue culture media (RPMI+20%LB and RPMI+10%FBS) recorded every 30 minutes over 24 hours. **(b-e)** Samples were loaded as triplicates, with average optical density every 30 minutes measured and graphed. Media only line indicates negative control. **(e)** RPIMI+10%FBS shows average OD of media containing bacteria, negative control and the difference between the two data points.

In order to further optimize the growth of *B. cenocepacia* in RPMI, we hypothesized that growth would be enhanced with the addition of bicarbonate. Standard RPMI buffered at 24mM $HCO3^-$ was compared to RPMI buffered at 48mM $HCO3^-$. A fresh plate with new colonies was prepared from the stock solution for the overnight cultures. Fresh medias were prepared to avoid contamination. The bacteria were diluted to a starting OD of 0.2 in each media shown in **Table 1.** The OD 600nm of the samples were manually checked at time 0, 4hr, 6hr and 18hr. **Figure 4** shows excellent growth of *B. cenocepacia* in all 6 media conditions tested. There was no major difference of growth of BCC in RPMI at 24mM HCO3 or 48 mM HCO3.

Media	0hr	2hrs	4hrs	6hrs	18hrs
LB	0.2	0.829	1.01	1.108	1.115
CA-MHB	0.2	0.674	0.885	1.028	1.153
RPMI + 20% LB	0.2	0.846	1.145	1.209	1.218
RPMI + 20% LB + 10% FBS	0.2	0.684	1.01	1.142	1.167
RPMI + 48 mM HCO3	0.2	0.7	0.966	1.023	1.004
RPMI + 48 mM HCO3 + 20% LB	0.2	0.781	1.142	1.181	1.135

Table 1: Manual Growth Assay of *B. cenocepacia* in various media conditions. Comparative growth assay in LB, standard bacteriologic media (CA-MHB) and supplemented RPMI. The starting optical density at time points 0 was standardized to 0.2. The OD was re-measured at 2hrs, 4hrs, 6hrs and 18hrs

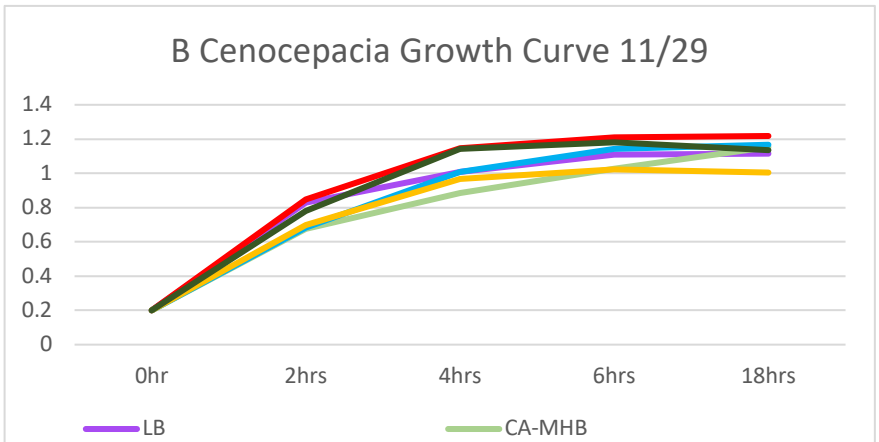


Figure 4: Manual Growth Curve Assay of *B. cenocepacia* in various media conditions. Data points from Table 1 were graphed to establish a growth curve of *B. cenocepacia* in various media conditions listed in the key.

Now that we were able to confirm adequate growth of *B. cenocepacia* in tissue culture media, we wanted compare MIC testing of azithromycin in conventional bacteriologic media (CA-MHB) and non-conventional tissue culture media. Based on the results from our previous experiments, we decided to narrow the tested medias to CA-MHB and supplemented RPMI (RPMI+20% LB and RPMI(48mM HCO3)+20%LB). Test plates were prepared as described in the methods above and incubated overnight with continuous shaking at 37˚C. The plates were read at A600nm after 24 hours. The average percent bacterial survival and the MIC in each media was calculated and is shown in **Table 2.** Using CA-MHB, the media used in standard antibiotic susceptibility testing, it appeared that Azithromycin does not exhibit adequate killing of BCC with a minimal inhibitory concentration of 64 µg/mL. On the other hand, testing performed with tissue culture media (RPMI) showed markedly increased activity of Azithromycin against BCC with an MIC of 0.25 µg/mL supporting our initial hypothesis.

CA-MHB			RPMI + 20% LB			RPMI @ 48 mM HCO3- + 20% LB	
Azithromycin Concentration	Average Percent Bacterial Survival		Azithromycin Concentration	Average Percent Bacterial Survival		Azithromycin Concentration	Average Percent Bacterial Survival
64 µg/mL	0.006		64 µg/mL	0.004		64 µg/mL	0.003
32 µg/mL	0.061		32 µg/mL	0.003		32 µg/mL	0.007
16 µg/mL	0.091		16 µg/mL	0.003		16 µg/mL	0.002
8 µg/mL	0.102		8 µg/mL	0.002		8 µg/mL	0.001
4 µg/mL	0.127		4 µg/mL	0.002		4 µg/mL	0.004
2 µg/mL	0.185		2 µg/mL	0.009		2 µg/mL	0.001
1 µg/mL	0.321		1 µg/mL	0.003		1 µg/mL	0.003
0.5 µg/mL	0.765		0.5 µg/mL	0.003		0.5 µg/mL	0.002
0.25 µg/mL	0.822		0.25 µg/mL	0.004		0.25 µg/mL	0.002
Positive Control	1		Positive Control	1		Positive Control	1
Negative Control	0		Negative Control	0		Negative Control	0
MIC	**64 µg/mL**		**MIC**	**0.25 µg/mL**		**MIC**	**0.25 µg/mL**

Table 2: Comparison of MIC of Azithromycin in bacteriologic media (CA-MHB) and tissue culture media (RPMI). The average percent bacterial survival was calculated by comparing the optical density of the bacteria at each antibiotic concentration versus the positive control. The minimal inhibitory concentration of Azithromycin in each media is highlighted in yellow.

In order to furthest assess bactericidal activity of azithromycin against *B. cenocepacia,* Resazurin was then added to the incubated samples. Active bacterial cells reduce the non-fluorescent resazurin (blue) to fluorescent resorufin (pink) and is therefore an indication of living bacteria. The color changes of the samples correlate with amount of active bacteria present in each sample. In CA-MHB the azithromycin bactericidal activity was concentration dependent with poor killing of BCC as seen by the of oxidation of resazurin by active bacteria even at high concentrations of azithromycin. In both RPMI medias, azithromycin exhibited increased bactericidal activity against BCC as there was lack of oxidation of resazurin indicating a lack of active bacteria. Of note, there is oxidation of one of three of the negative controls (that did not have bacteria), but this is likely attributed to human error in preparation of the plates **(Figure 5)**.

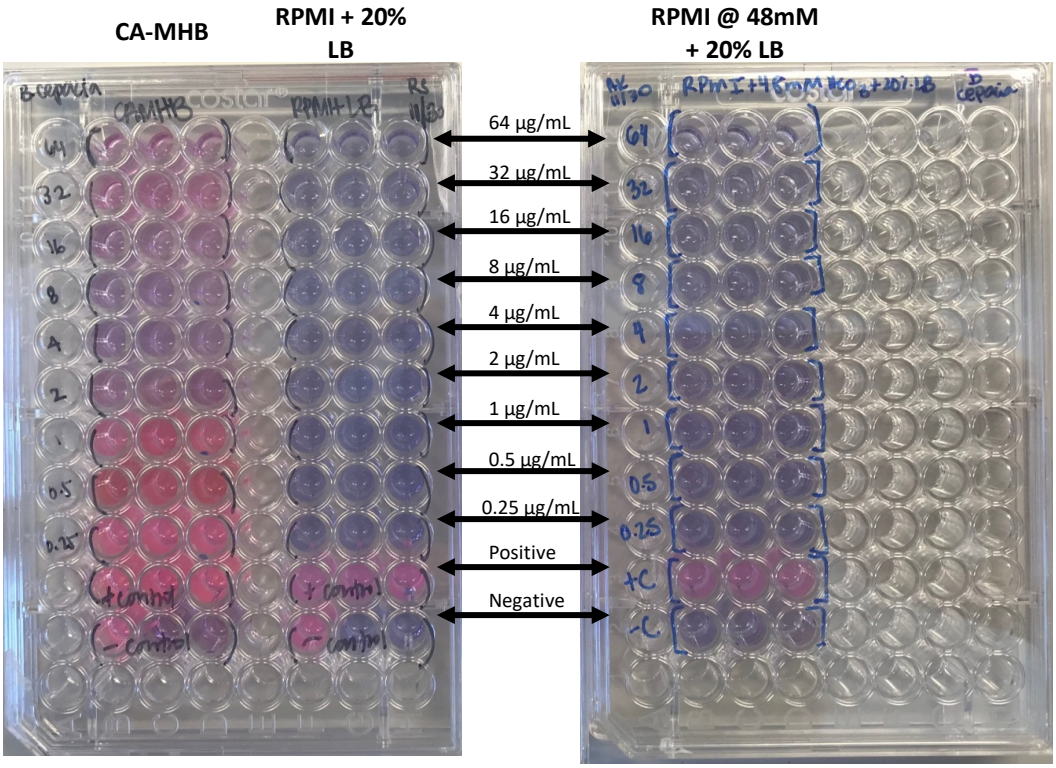


Figure 5: Comparison of Bactericidal Activity of Azithromycin in CA-MHB versus RPMI. Azithromycin bactericidal activity comparison in standard bacteriologic media (CA-MHB) and tissue culture media (RPMI+20%LB and RPMI@48mM $HCO3^-$ + 20%LB). Samples appear in triplicates with decreasing azithromycin concentration. Positive control contain bacteria and no antibiotics. Negative controls contain only media. Resazurin oxidation to pink color is used to indicate active living bacteria. Lack of oxidation (blue) indicates lack of living bacteria.

Discussion:

Our preliminary data shows a possible bactericidal effect of azithromycin against BCC when tested in tissue culture media versus standard bacteriologic media. However, unfortunately, this experiment was repeated multiple times and we were not able to consistently reproduce the initial promising results. Repeat experiments sometimes failed to show growth of the BCC in the tissue culture media that served as positive controls. This is likely due to the small volume of the bacteria plated in the wells and not having defined the optimal conditions/supplements in the tissue culture media for these highly fastidious bacteria. Other factors contributing to the lack of growth might include temperature fluctuations during transport to and from the incubator as well as strain-specific growth limitations within this isolated strain. Also, equally likely is the possibility that the tissue culture media, RPMI, specifically used in these experiments does not contain the essential nutrients required for BCC growth in small-volume wells.

To further explore the contributions of these factors, we plan to attempt the same experiment with a different isolated strain of BCC. We also plan to attempt this experiment using a different tissue culture medium, supplemented-DMEM, which differs from RPMI in that it contains more nutrients to enhance bacterial growth. In addition, attempts will be made to supplement the RPMI media with FBS, BHI, LB, and bicarbonate. From these experiments we hope to determine which factors contributed the lack of BCC growth in small-volume RPMI wells in our repeat experiments and develop a replicable methodology.

Following these modifications, in order to demonstrate the synergistic effect of azithromycin with innate immune system peptides such as LL-37 and colistin, we will add these peptides when calculating the MIC of azithromycin to test this hypothesis. As mentioned in the introduction[13], previous experiments have demonstrated the synergy between innate immune system peptides, specifically LL-37 and colistin against other gram negative bacteria. If our hypothesis is supported these experiments, it would indicate that a similar synergistic effect is present in azithromycin's interactions with LL-37 and colistin against BCC.

Ultimately, this conclusion could lead to the development of clinical trials to test the effectiveness of azithromycin against BCC *in vivo*. If such efficacy could be demonstrated, it could very well lead to the development of new antibiotic combinations against multi-drug resistant bacteria, such as BCC. Such innovations would impact treatment protocols for BCC colonization and/or exacerbations in CF patients as well as other populations. In light of the increasing prevalence of multi-drug resistant organisms in CF patients, immunocompromised populations, and others, a new strategy to combat these diseases would be of tremendous importance.

However, there are several limitations to this optimistic proposal. Although possible modifications as suggested above might allow the results to be reproduced, appropriate parameters have not yet been determined. In addition, our experiment utilized a single species of BCC, *B. cenocepacia*. Although this species is the most prevalent, other species, such as *B. multivorans* and *B. gladioli*, are also involved in colonization and would possibly alter the interaction of azithromycin in vivo. Another clear limitation of this study is, of course, the fact that it was conducted in vitro. In vivo clinical trials would be necessary to examine other possible factors that impact the synergy between azithromycin and innate immune peptides within live patient populations.

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