Screening for Predicted Beta-lactamases within *Elizabethkingia anopheles*

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Abstract: *Elizabethkingia* causes meningitis and sepsis among the immunocompromised and neonates. Many infections of *Elizabethkingia* are associated with high mortality rates and a significant rate of infection. Its high mortality rates are due to the bacterium's resistance to almost all antibiotics typically used to treat gram negative infections, such as ampicillin and tetracycline. This resistance is achieved by *Elizabethkingia's* production of beta-lactamases, which work by hydrolyzing or cleaving the components of beta-lactam antibiotics. In this experiment, beta-lactamase genes of *Elizabethkingia anopheles* were screened for the beta lactamase characteristic of antibiotic resistance. Unfortunately, based on the results obtained, it can be concluded that genes Agl 2045 and 2038 are likely not beta-lactamases, as the average inhibition zone of each antibiotic tested fell well within what one would deem susceptible. It is suspected that genes Agl 2038 and 2045 play a role in the peptidoglycan synthesis pathway, but further research must be conducted to confirm this.

Keywords: Bacteria, Antibiotics, *Elizabethkingia*, Beta-Lactamases

Introduction

Elizabethkingia is a genus of bacteria that is differentiated from its former genus of *Chryseobacterium* by its unique 16S rRNA gene sequence (Kim et al. 2005). The bacteria are gram negative rods that are non-spore forming and non motile. (Erikson et al. 2017). *Elizabethkingia* causes meningitis and sepsis among the immunocompromised and neonates (Lau et al 2016, Hsu et al. 2011, Lin et al. 2009). and is found in all parts of the environment (Kämpfer et al. 2012). Many infections of *Elizabethkingia* are associated with high mortality rates and a significant rate of infection (Hsu et al. 2011, Lau et al. 2016). Its high mortality rates are due to the bacterium's resistance to almost all antibiotics typically used to treat gram-negative infections, such as ampicillin and tetracycline (Kämpfer et al. 2012). This resistance is achieved by *Elizabethkingia's* production of beta-lactamases, which work by hydrolyzing or cleaving the components of beta-lactam antibiotics. Beta-lactam antibiotics are penicillin and all the generations derived from penicillin, and affect the bacteria by binding to penicillin binding proteins (PBPs) (Heesemann 1993). PBPs are involved in cell wall synthesis, so when beta-lactams bind to them, the cell wall is weakened and the bacterium dies due to osmotic instability. Beta-lactam antibiotics are classified by their amino acid sequences, with each having a core beta-lactam ring among them (Heesemann 1993). There are many beta-lactams that are found in the world naturally, but a need for a greater variety of the drug in medicine led to the production of synthetic beta-lactams as well. Currently, under the RAST database, there are 21 beta lactamases encoded among the species of *Elizabethkingia anophelis* in terms of their gene sequence. To fully understand these genes, we must further understand and characterize their reactions to beta-lactam substrates and inhibitors. The

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immediate question that this experiment strove to answer was how beta-lactamase producing genes, which produce the phenotype of resistance, respond to certain beta-lactam antibiotics.

Methods

Antibiotic resistance screening was done using the Kirby- Bauer Disk Diffusion method. Before this experiment began clones of the proteins in question were made in the previous semester, and then frozen into vials. When this experiment began, the vials were defrosted and the bacteria was grown up onto plates. The bacteria were grown by streaking the plates in order to grow distinct colonies. The first step in streaking is to turn on a Bunsen burner in order to keep the work area sterile. The inoculation loop is then sterilized by running it through the Bunsen burner at a 45° angle three times, waiting each time for the metal to start turning red before pulling it out of the flame. Once the loop has cooled off it is dipped into the vial of bacteria. When the loop appears to have liquid in its center the plate can be quadrant struck. These steps are then repeated for each additional vial of bacteria. When all of the plates have been streaked they are then placed into the incubator overnight.

Next, the plates of bacteria were gram stained. Gram staining is done to check if any contamination has taken place. Since *Elizabethkingia* is a gram-negative bacteria it will be completely decolorized and appear pink in coloration if uncontaminated. A Gram stain is done by sterilizing the inoculation loop, as indicated above, and smearing the loop into the culture that was grown up. 55 micro liters of distilled water are then placed onto the microscope slide. The culture is then smeared onto the slide using the inoculation loop. The slide is then left to sit until dry. Crystal violet is then added to the slide to cover the entire smear of bacteria. The slide is left to sit one minute before it is washed off with distilled water. Gram's iodine is then deposited onto the slide until it completely covers the slide and it too is left on for a minute. The Gram's iodine is washed off with ethanol until the culture on the slide runoff is completely decolorized. Safranin is then added to the slide until the smear is covered, left to sit for one minute, and then is washed off with distilled water. The slide is then placed under a microscope to see if any contamination has occurred. Contamination is easily spotted, as the purple color will of the crystal violet will stick to any Gram-positive bacteria, making it easily

Table 1 - Agl 2045 and Skb3 zones of susceptibility after 24 hours, N=3, N=2

IPTG Concentration 24 hr[a]	Agl 2045 w/ Penicillin	Skb3 w/ Penicillin[c]	Agl 2045 w/ Ampicillin	Skb3 w/ Ampicillin[c]
0	14.50±0.71mm[c]	15.00±0mm	25.00±0mm[c]	26.00±1.41mm
0.1	17.30±1.53mm[b]	17.00±0mm	30.67±2.31mm[b]	30.00±0mm
0.25	16.30±1.15mm[b]	17.5±0.71mm	30.00±0.71mm[b]	30.00±0mm

[a] Concentration in µg/mL
[b] N=3
[c] N=2

Table 2 - Agl 2045 and Skb3 zones of susceptibility after 48 hours, N=3, N=2

IPTG Concentration 48 hr[a]	Agl 2045 w/ Penicillin	Skb3 w/ Penicillin[c]	Agl 2045 w/ Ampicillin	Skb3 w/ Ampicillin[c]
0	14.50±1.53mm[c]	15.50±0.71mm	25.50±5.51mm[c]	24.50±3.54mm
0.1	18.00±1.73mm[b]	18.50±0.71mm	30.67±1.53mm[b]	30.00±1.41mm
0.25	16.67±1.53mm[b]	16.50±0.71mm	31.00±1.73mm[b]	30.00±0mm

[a] Concentration in µg/mL
[b] N=3
[c] N=2

Table 3 - Agl 2038 and Skb3 zones of susceptibility after 24 hours, N=3

IPTG Concentration 24 hr[a]	Agl 2038 w/ Piperacillin[b]	Skb3 w/ Piperacillin[b]	Agl 2038 w/ Imipenem[b]	Skb3 w/ Imipenem[b]
0	34.67±16.97mm	31.33±0.58mm	31.00±1.73mm	30.00±5.77mm
0.1	36.33±12.49mm	33.67±5.29mm	34.00±2.65mm	33.00±8.50mm
0.25	36.33±2.31mm	37.33±4.51mm	37.33±2.31mm	37.33±2.52mm

[a] Concentration in µg/mL
[b] N=3

Table 4 - Agl 2038 and Skb3 zones of susceptibility after 48 hours, N=3

IPTG Concentration 48 hr[a]	Agl 2038 w/ Piperacillin[b]	Skb3 w/ Piperacillin[b]	Agl 2038 w/ Imipenem[b]	Skb3 w/ Imipenem[b]
0	33.33±4.04mm	32.33±4.16mm	32.33±2.52mm	28.67±0.58mm
0.1	35.67±4.58mm	37.00±7.23mm	34.00±3.21mm	33.33±13.89mm
0.25	37.67±4.93mm	40.00±3.06mm	35.00±3.00mm	35.33±2.65mm

[a] Concentration in µg/mL
[b] N=3

distinguished from the Gram-negative bacteria.

After Gram Staining to confirm that the bacteria has not been contaminated, the process of antibiotic screening by the Kirby-Bauer Disk Diffusion method can begin. The first step in this process is selecting an isolated colony from the plate of bacteria growth. Then, using a micropipette tip, the isolated bacterial growth is picked up and placed into a test tube of media. The test tube is then placed into the incubator for 24 hours in order to grow. After 24 hours the tube of media is inoculated using 5 micro liters of IPTG and placed back into the incubator for another hour and a half. Once an hour and a half has passed the tube of media is diluted down to an absorbance of 0.1by using the mass spectrometer. The diluted media can then be streaked onto plates. Streaking the diluted media requires a Bunsen burner to be lit in order to keep the work area sterile. Cotton swabs are then dipped into the diluted media and swabbed onto plates, making sure that media has been swabbed on all parts of the plate. After all of the plates have been swabbed antibiotic disks

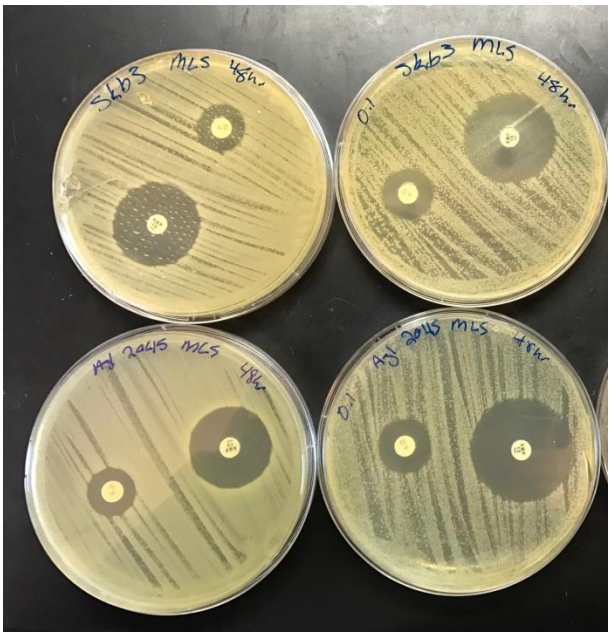

Figure 1 - Examples of Agl 2045 plates containing zones of clearance

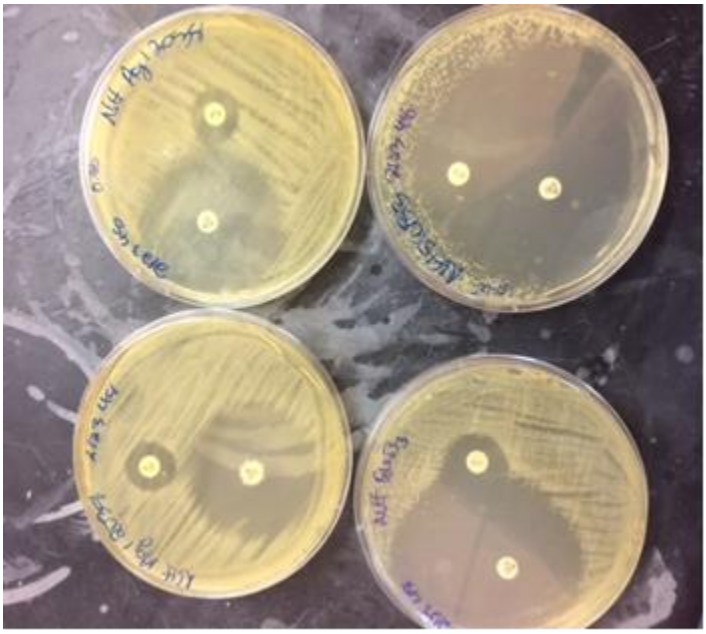

Figure 2 - Examples of Agl 2038 plates containing zones of clearance

are placed onto each plate. The plates are then incubated for 24 hrs. At the 24-hour mark the plates are taken out of the incubator and the zones of inhibition of each antibiotic are measured in millimeters and recorded. The plates are placed back into the incubator for another 24-hours. At the 48-hour mark the zones of inhibition are again measured and recorded.

Results and Discussion

When this project began it was initially to screen for predicted beta-lactamases. The lab in which this project was conducted had previously cloned numerous suspected beta-lactamases into *E. coli*. The RAST database had predicted over 21 beta-lactamases between the three species of *Elizabethkingia*: *anophelis*, *meningoseptica*, and *miricola*. Research continued this semester by starting the screening process for each predicted gene. For a gene to be considered a beta-lactamase, it must display a resistance to penicillin and penicillin-derived antibiotics. For both genes (Agl 2045, Ag1 2038) and pSKB3 (the empty vector), the average zone of clearing created by the antibiotic discs (imipenem, piperacillin, ampicillin, and penicillin, respectively) were all in the range of susceptibility (Tables 1-4). The borderline zones of susceptibility for each antibiotic are as follows- imipenem: diameter of zones ≥16 mm, piperacillin: diameter of zones ≥21 mm, ampicillin: diameter of zones ≥17 mm, and penicillin: diameter of zones ≥15 mm. Unfortunately, based on the results obtained, it can be concluded that genes Agl 2045 and 2038 are likely not beta-lactamases, as the average inhibition zone of each antibiotic tested fell well within what one would deem susceptible. It is believed that these genes play separate roles within the cell, with Agl 2038 likely playing a role in the peptidoglycan synthesis pathway (a pathway used in the building of the cell wall). In fact,

the RAST database has already reclassified it as such. It has been hypothesized that Agl 2045 is also playing a role within the peptidoglycan synthesis pathway, although the RAST database has yet to reclassify Agl 2045. What could be determined about Agl 2045 is that it is an enzyme within *Elizabethkingia anophelis* that is being produced, though exactly what its role is has yet to be determined. Both of these hypotheses would need to be researched further in order to obtain any conclusive information.

There are many different paths that this research could take to further characterize these genes. The first step in trying to classify both Agl 2038 and Agl 2045 would be protein purification. From this point, research could be conducted on multiple enzymatic assays to further characterize the role they play within *Elizabethkingia anophelis*. Once the process of running the enzymatic assays are completed, crystallization could be carried out to look at the structures of the two genes (Agl 2038, Agl 2045), compare them to already known structures, and look for unique characteristics.

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