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Molecular Analysis of β-Lactamase Genes in Antibiotic Resistant Bacteria

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One of the biggest medical milestones has been the discovery and use of antibiotics to treat deadly infectious diseases. Antibiotics are drugs that have the capability of blocking essential processes and mechanisms in bacteria that will either stop them from dividing or kill them. There are also a wide range of different antibiotics in terms of the types of bacteria they can target and how they go about stopping and destroying the bacteria (What are antibiotics, 2012). The discovery of penicillin in the late 1920's marked the beginning of finding and using antibiotics to treat various bacterial diseases that had no previous treatments. It was a huge turning point in medical history since up until the late 20's one of the leading cause of death was due to infectious diseases such as pneumonia and tuberculosis.

However, with the wide use of antibiotics to treat infections and diseases, it did not take long for bacteria to gain resistance to these antibiotics and leave them ineffective. Bacteria not only have the ability to divide rapidly they also utilize horizontal gene transfer in order to share and gain new genetic information through the environment. As each new generation arises, the previous ones can pass down evolutionary modifications that will allow the new generation to survive, thrive and ultimately replicate new generations while also still obtaining new information from their surroundings. This is how antibiotic resistance came to be with the continuous use of antibiotics. Each time a bacterial strain was exposed to antibiotics, the new generations would gain new mutations that were selective against the antibiotics which would allow for them to continue to grow and divide in the presence of the antibiotics (Aminov, 2010). These selective bacteria are resistant to the antibiotics and will continue to produce new generations that are also resistant, rendering the antibiotics useless against the bacteria.

Since the late 1980's, no new antibiotics have been found and so now we face a crisis of not being able to treat disease because of the resistance they have built up to our antibiotics. One of

the main reasons for the growth of antibiotics resistance is the overuse of antibiotics. Many times, it has been the case that antibiotics have been prescribed to patients who did not need antibiotics, such as for viral infections or because the patients wanted them. There is also the problem that when antibiotics are prescribed for an infection, people will not finish their prescription because they think they are all better. All this does is introduce partial resistance to the few bacteria that are still in their systems which will then divided, build up, and pass on the antibiotic resistance. Another major reason for the antibiotic resistance problem is the use of antibiotics in agriculture (Witte, 2013). Antibiotics are added in low doses to the food that is fed to farm animals to prevent diseases in them and to also help them grow bigger. When the animals are then slaughtered and sold to the public, people will then eat their meat which contains low dose amounts of these antibiotics which will lead to the development of antibiotic resistance (Scott, 2009).

The aim of this research project was to develop a model system to look at the development of antibiotic resistance in bacteria. The focus was on bacteria that are resistant to β-lactam antibiotics, one of the broadest and most widely used class of antibiotics. In the cases of to β-lactam antibiotic resistance, the production of the β-lactamase enzyme is responsible for the disruption of the bonds in the β-lactam ring that is found in the β-lactam group of antibiotics which render the antibiotic useless (Poole, 2004). The β-lactamase enzyme is usually carried on a plasmid, a mobile vector that can code for genes involved in antibiotic resistance. One of the biggest concerns with plasmids being involved in antibiotic resistance is that plasmids are able to replicate and pass on their information easily and have great mobility. There is also the fact that plasmids can move from different hosts, in this case among different bacterial strains is of great

importance and concern since it makes it much easier for resistance to spread among the bacterial community (Smillie, 2010).

The other aim of this project was to look at isolated bacterial samples, specifically form gram-negative bacteria where β-lactamase production is usually found, and look for how prevalent it is in the environmental samples and look to identify how much heterogeneity there is. The samples are then sequenced to determine the species of each of the samples tested.

Material and Methods

This project was conducted by first collecting various environmental feces samples from assorted farm animals such as llamas, sheep, goats and donkeys. A fecal sample from a bearded dragon kept as a pet was also obtained to use as a comparison from an indoor animal to a common agriculturally raised one.

The fecal sample from the bearded dragon was diluted 100-fold and plated on a MacConkey +Ampicillin plate and incubated overnight. From that plate, 40 isolated colonies were selected and patch plated on various plates to test of antibiotic resistance (MacConkey, MacConkey +Ampicillin (AMP), LB +Kanamycin (KAN), LB+ Chloramphenicol (CM), and LB+ Tetracycline (TET)). Four colonies were then selected for chromosomal DNA extractions. DNA was extracted using a spooling technique. The DNA samples were then used to run PCR for 16s using VENT polymerase along with as CTX1 and CTX2 primers. Samples were then sent out for sequencing.

The fecal samples from the farm animals were previously plated on LB plates. 25 samples were selected and streaked for isolation on MacConkey +Ampicillin plates. Of the 25, only 11 grew which were then plated on LB to test for antibiotic resistance. Five antibiotic discs were placed

on each plate for each sample (Piperacillin/Tazobactam (TZP), Piperacillin (PIP), Ticarcillin (TIC), Ticarcillin with Clavulanic acid (TIM), Ampicillin with Sulbactam (SAM)). After a two day incubation period, the zones of inhibition were measured for each disc for each of the samples. Five of the samples, which were not resistant to TIC, were selected and tested in triplicates for TIC and DNA extraction was done for each of the samples. Triplicates were then also done for each of the other antibiotics tested to see if resistance to them changed over time. DNA was then used to run PCR on 16s using Taq polymerase and then sent out for sequencing.

Results

Bearded Dragon Samples

When the samples were each plated on the different plates to test for antibiotic resistance, many of the samples did not grow on the LB+ Kanamycin plates and all grew on the MacConkey+ Ampicillin plates. Many of the colonies were very mucosal in nature and most were pigmented a bright to dark ink color. Figure 1 summarizes the results for each of the samples.

Figure 1: Results and classification of the 40 bearded dragon samples.

	Mac	Mac+Amp	LB+Kan	LB+CM	LB+TET
1	+/+; doesn't seem to be E.	+/-	+/+	+/+	+/+
2	+/+;	+/+	+/-	+/-	+/+
3	+/+	+/+	+/+	+/-	+/+
4	+/+	+/+	+/+	+/+	+/+
5	+/+; heavily pigmented	+/+	+/+	+/+	+/+
6	+/+	+/+	+/+	+/+	+/+
7	+/+; heavily pigmented	+/+	+/+	+/+	+/+
8	+/+	+/+	+/+	+/+	+/+
9	+/+	+/+	+/+	+/-	+/+
10	+/+; heavily pigmented	+/+	+/+	+/+	+/+
11	+/+	+/+	+/+	+/-	+/+
12	+/+; heavily pigmented	+/+	+/+	+/+	+/+
13	+/+	+/+	+/+	+/+	+/+
14	+/+; heavily pigmented	+/+	+/+	+/+	+/+
15	+/+	+/+	+/-	+/+	+/+
16	+/+	+/+	+/+	+/+	+/+
17	+/+	+/+	+/+	+/+	+/+
18	+/+	+/+	**+/-**	+/+	+/+
19	+/+; doesn't seem to be E.	+/+	+/+	+/+	+/+
20	+/+; heavily pigmented	+/+	+/+	+/+	+/+
21	+/+	+/+	+/+	+/-	+/+
22	+/+; heavily pigmented	+/+	+/+	+/+	+/+
23	+/+	+/+	+/+	+/+	+/+
24	+/+	+/+	+/+	+/+	+/+
25	+/+	+/+	+/+	+/+	+/+
26	+/+	+/+	**+/-**	+/+	+/+
27	+/+	+/+	+/+	+/+	+/+
28	+/+	+/+	+/+	+/+	+/+
29	+/+; heavily pigmented	+/+	+/+	+/+	+/+
30	+/+	+/+	+/+	+/+	+/+
31	+/+; heavily pigmented	+/+	+/+	+/+	+/+
32	+/+; doesn't seem to be E.	+/+	+/+	+/+	+/+
33	+/+	+/+	**+/-**	+/+	+/+
34	+/+	+/+	**+/-**	+/+	+/+
35	+/+	+/+	+/+	+/+	+/+
36	+/+; heavily pigmented	+/+	+/+	+/+	+/+
37	+/+	+/+	+/+	+/+	+/+
38	+/+	+/+	+/+	+/+	+/+
39	+/+; heavily pigmented	+/+	+/+	+/+	+/+
40	+/+	+/+	+/+	+/+	+/+

Figure 1: A +/+ indicates that there was growth present, a +/- indicates that there was some growth present, but most likely resistant. The beige coloring indicates the colonies that were very mucosal in nature, pink is those that are heavily pigmented, and green are those that don't appear to be *E. coli*.

Through DNA sequencing, it was found that the bearded dragon samples were *Citrobacter freundii*. Further antibiotic testing of these samples was not possible due to the loss of sample vitality.

Farm Animal Samples

The 11 samples that were used for antibiotic testing began to show signs of antibiotic resistance occurring as the testing progressed. Figure 2 shows the measurements for the zones of inhibition. Antibiotic resistant was observed occurring for a number of reasons. In sample D3-3, isolated colonies were seen growing on the outer perimeter of the inhibition zones for the PIP and TIM discs. Similarly, in the A4-5 sample, colonies were seen growing around the TZP and PIP disc zones. The CALF-1 sample had a very small zone of inhibition around the PIP discs which suggests resistance.

Figure 2: Measurements of zones of inhibition for farm animal samples.

	TIC	PIP	SAM	TZP	TIM
A4-1	RS	1.5	1.4	2.2	2.1
D2-3	RS	1.5	1.9	2.3	2
A4-5	0.9	2.2	RS	2.1	1.8
A4-6	RS	1.7	1.8	2.4	2.1
CALF-1	RS	0.7	1.2	2	1.7
D1-2	RS	1.4	1.5	2.1	2.1
A2-3	0.9	1.8	1.4	1.9	2
D3-3	2.1	1.8	RS	2.2	2.2
D3-1	1.6	2	1.7	2.2	2.2
CALF-2	RS	1.4	1.4	2.2	1.8
LLAMA-3	1.2	2	1.1	2	1.5

Figure 2: Summary of the zones of inhibition measured for each of the antibiotic discs. Measurements were done in cm.

Those that were not resistant to TIC (5 samples) were selected for further testing. A TIC triplicate was done on the samples to ensure that resistance had not occurred yet (Figure 3-A). The A2-3 sample started to show signs of developing resistance after one day of incubation, but

the other samples all maintained their sensitivity to the antibiotic. On day two after incubation, the A2-3 sample showed total resistance to TIC (Figure 3-B). A mean and standard deviation was calculated for each of the samples (Figure 4).

Figure 3: Triplicate results for TIC antibiotic.

A.

DAY 1	1	2	3
A2-3	RS	0.8	0.8
A4-5	1.4	1.3	1.4
LLAMA 3	1.2	1.5	1.4
D3-3	2.3	2.3	2.2
D3-1	1.6	1.6	1.6

B.

DAY 2	1	2	3
A2-3	RS	RS	RS
A4-5	1.4	1.3	1.3
LLAMA 3	1.2	1.4	1.4
D3-3	2.3	2.3	2.2
D3-1	1.6	1.6	1.6

Figure 3: Measurements for the zones of inhibition for the 5 samples that were not initially resistant to TIC. Table A shows the measurements after one day of incubation while table B shows the measurements after two days of incubation. Measurements were done in cm.

Figure 4: Mean and standard error for the inhibition zones produced by TIC antibiotic.

	Mean	SD
A2-3	0	0
A4-5	1.333333	0.04714
D3-1	1.6	2.22E-16
D3-3	2.266667	0.04714
llama 3	1.333333	0.094281

Figure 4: Measurements done in cm for the mean.

Triplicates were then also done for each of the other antibiotics being testes (Figure 5) and a mean and standard deviation was also calculated for each sample. Almost all of the samples started to show growing resistance to the antibiotics. A2-3 was showing resistance to PIP and TZP with the zones appearing weak. A4-5 showed colonies growing in the zones for both PIP, TIM and TZP. D3-3 also showed isolated colonies growing in the zones for TIM and PIP.

Figure 5: Triplicate results for each antibiotic tested.

	PIP	PIP	PIP	Mean	SD
A2-3	1.8	1.2	1.7	1.566667	0.262467
A4-5	2.2	2.2	2.2	2.2	0
D3-1	2.2	2.1	2.2	2.166667	0.04714
D3-3	2	1.9	2.1	2	0.08165
llama 3	2.5	2.4	2.6	2.5	0.08165

	SAM	SAM	SAM	Mean	SD
A2-3	1.3	1.3	1.4	1.333333	0.04714
A4-5	RS	RS	RS	0	0
D3-1	1.8	1.8	1.9	1.833333	0.04714
D3-3	RS	RS	RS	0	0
llama 3	1.2	1.2	1.2	1.2	0

	TZP	TZP	TZP	Mean	SD
A2-3	1.9	1.9	1.9	1.9	2.22E-16
A4-5	2.1	2.1	2.2	2.133333	0.04714
D3-1	2.2	2.4	2.4	2.333333	0.094281
D3-3	2.5	2.3	2.2	2.333333	0.124722
llama 3	2.5	2.6	2.4	2.5	0.08165

	TIM	TIM	TIM	Mean	SD
A2-3	2.4	2.1	2.2	2.233333	0.124722
A4-5	2	1.8	1.9	1.9	0.08165
D3-1	2.5	2.6	2.6	2.566667	0.04714
D3-3	2.3	2.3	2.3	2.3	0
llama 3	2.6	2.4	2.4	2.466667	0.094281

Figure 5: Measurements for the zones of inhibition for each antibiotic along with the mean values and standard deviation for each sample. Measurements were done in cm.

PCR and DNA sequencing results showed that the five samples tested were all *E. coli*.

Discussion

The overall results of these experiments show how antibiotic resistance can spread and develop over generations in bacterial populations. In looking at the farm animal samples that were tested with the TIC antibiotic, it was seen that just in one day resistance can occur. This can be attributed to the antibiotic resistance genes being contained on a plasmid that is able to pass down and transport the gene to other colonies and even different bacterial species (Smillie, 2010).

In looking at the antibiotic resistance seen between the domesticated bearded dragons and the farm animals, we can see that in both, resistance is prevalent. This is something to worry about since it shows that antibiotic resistance can occur anywhere and we as people are exposed to it

all the time. This can be a sociological issue due to its implications on the health of people and the food that we eat. With the increasing use of antibiotics in livestock to keep animals healthy and prevent diseases, leads to humans being exposed to these antibiotics and do can then lead to antibiotic resistance developing. This is particularly a concern for antibiotics that are used both for agricultural use as well as for human use in treating diseases and infections (Schmidt, 2002). This is especially of concern for the workers of the farms that raise and treat the animals for food production. There is also then the concern about agricultural runoff from the farms into local towns and water supplies. As seen from the experiments conducted, the feces from the farm animals has multiple strains of bacteria that are present that are antibiotic resistant. These bacteria are then fed into the ground and can end up in local streams and rivers when it rains and can find their way to our drinking water and homes.

This has become a global health issue as well as an economical issue. Livestock and antibiotic use is something that occurs globally and with so many different antibiotics being used and the threat of transmitting disease and infections grows with people traveling and antibiotic resistance being on the rise, its implications pose a great economical concern (Rudholm, 2002). The cost of antibiotic treatments in animals must be weighed against the cost of those treatments in humans as well as the medical implications if humans contract diseases and infections that do not respond to any antibiotics. There is also the implication that those that are most at risk for suffering from antibiotic resistance issues are those from the lower socioeconomic classes that do not have access to reliable medical care. This class of people are more likely to live in areas with conditions that could expose them to more antibiotic resistant bacteria. There is also the issue with how available antibiotics are. Consumers can simply go online and buy their own antibiotics if they wanted to which can be very dangerous and makes it much harder to control

the use of antibiotics. These are the questions that need to be asked and the concerns that need to be thought about as the use of antibiotics continues and resistance rises.

Conclusion

The overall findings of this research experiment show the concerns that are associated with antibiotic resistance and show how it can occur easily. It also shows the prevalence of antibiotic resistance around us and how it can be found in various settings. The findings of this research offer room for additional research and implications. Other bacterial species can be tested and looked for in samples as well as widening the sample pool and possibly getting samples from humans as well to see how the animal and agricultural samples compare to us.

References

Aminov, R. I. (2010). A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Front. Microbio. Frontiers in Microbiology, 1*. doi:10.3389/fmicb.2010.00134

This journal article discusses the discovery of antibiotics and how it was a turning point for infectious disease for about 20 years. It then addresses the problem with antibiotic resistance and how there are hardly any new antibiotics being discovered today. It also discusses the complexity of the antibiotic resistance problem and how we should go about preventing it.

Monstein, H.-J., Å. Östholm-Balkhed, M. V. Nilsson, M. Nilsson, K. Dornbusch, and L. E. Nilsson. "Multiplex PCR Amplification Assay for the Detection of Bla SHV, Bla TEM and Bla CTX-M Genes in Enterobacteriaceae." *Apmis* 115.12 (2007): 1400-408. Web. 2 May 2016.

This paper provides much of the background and basis for this project by providing information on the primers that will be used to categorize and analyze the samples that are collected. The primers used will allow us to not only isolate specific genes but will also assist in the phenotypic characterization of them.

Poole, K. "Resistance to β-lactam Antibiotics." *CMLS, Cell. Mol. Life Sci. Cellular and Molecular Life Sciences* 61.17 (2004). Web. 1 May 2016.

Rudholm, N. (2002). Economic implications of antibiotic resistance in a global economy. *Journal of Health Economics, 21*(6), 1071-1083. doi:10.1016/s0167-6296(02)00053-x

This article takes a look at the global economic implications of the antibiotic resistance issue and how it effects people globally. It also discusses and proposes possible ideas for the allocation of resources that would help in optimizing the economic issue brought upon this problem. It also addresses the fact that this is a global issue and how if one country decides to change its ways, it would ultimately not be beneficial on a global scale and all countries need to work together in order to create a beneficial change.

This paper discusses the origins and mechanisms by which beta-lactamase is able to resist the beta-lactam antibiotics. It breaks down how common beta-lactamase is in each of the various antibiotics, especially in those that are gram negative, since this resistance is virtually unseen in gram-positive microbes. It also proposes the development of new drugs that will inactivate novel target unrelated to antimicrobials.

Schmidt, C. W. (2002). Antibiotic resistance in livestock: more at stake than steak. *Environmental Health Perspectives*, *110*(7), A396–A402.

This article discusses the growing concerns about the growing antibiotic resistance issues and how it is tied to antibiotic use in agriculture and livestock. There are some antibiotics that are commonly used in livestock animals that are of little concern and are helpful in improving feed efficiency in the animals. However, there is much concern over the antibiotics that are used in both animals and humans. It also discusses the issues with how to regulate the use of antibiotics in animals and what the implications can be if antibiotics were to not be used.

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. C., & de la Cruz, F. (2010). Mobility of Plasmids . *Microbiology and Molecular Biology Reviews : MMBR*, *74*(3), 434–452. http://doi.org/10.1128/MMBR.00020-10

This paper provides information on what plasmids are and what their roles and functions are within cells. It also provides information on the mechanisms behind plasmid mobility and how important understanding its mobility is when it comes to gene transfer and antibiotic resistance. It also discusses the ability to use plasmids in order to trace and follow the evolutionary changes of conjugation elements in bacteria in order to better understand not only the plasmids mobility, but also the evolutionary changes among bacterial strains which will better allow us to see and understand how many of the antibiotic resistances are passed on.

Scott, Geoff. "Antibiotic Resistance." *Medicine* 37.10 (2009): 551-56. Web. 1 May 2016.

This article discusses the dangers of antibiotic resistance and how work should be done in order to prevent it. It also addresses the investments being done to find new antibiotics and suggests the return to older antibiotics. It also explains some of the molecular mechanisms involved in antibiotic resistance.

What are antibiotics and how do they work? (2012, April 5). Retrieved May 01, 2016, from http://www.nps.org.au/medicines/infections-and-infestations/antibiotics/for-individuals/what-are-antibiotics-and-how-do-they-work

This source provides a general overview of what antibiotics are and how and why they are use in the medical field in order to treat various infectious diseases. It also discusses how there are a variety of different antibiotics and how they can all work in different and various ways in order to inhibit bacteria.

Witte, W. (2013). Antibiotic resistance. *International Journal of Medical Microbiology, 303*(6-7), 285-286. doi:10.1016/j.ijmm.2013.06.003

This article talks about the dangers of antibiotic resistance and how resistance occurs at a low frequency and naturally which we now have a better understanding about thanks to genomics. It also talks about some of the mechanisms involved in antibiotic resistance such as horizontal gene transfer and intrinsic resistance. It also mentions some antibiotic resistant strains and discusses how they have become a problem not only in hospital setting but also in having origins with farm animals.