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Investigating Antimicrobial Properties of Snake Venoms Against B. cereus, B. subtilis, E. coli, and P. vulgaris

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Investigating antimicrobial properties of snake venoms against *B. cereus*, *B. subtilis*, *E. coli*, and *P. vulgaris*

by

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ABSTRACT

The increasing incidence of antibiotic resistant bacterial infections has provoked the attention of health officials and scientists as a major threat to global public health. Antibiotic resistance is the product of overprescription by doctors, ineffective or shortened dosing by the patient, and more. When a bacterial infection is ineffectively treated as such, persistent pathogenic cells are given the opportunity to proliferate and spread their resistance to other cells. With bacteria utilizing such tools to fight and ultimately resist our current treatment methods, investigation towards the next novel mechanism of inhibition is essential. Venom is of particular interest to many scientists as a potential antimicrobial for its numerous bioactive capabilities. Venoms from several marine animals, insects, reptiles, and others have been collected and analyzed for signs of antimicrobial activity and other potential pharmaceutical uses. We investigated the antimicrobial properties of six venoms harvested from snakes of diverse origin. Using the Kirby-Bauer disk diffusion assay, venom concentrations from 0.1-1000 μg/mL were applied and analyzed for signs of antimicrobial activity against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, and *Proteus vulgaris* by measuring zones of inhibition in centimeters (cm). For *B. cereus, E. coli* and *P. vulgaris*, results thus far have shown no inhibition, but for *B. subtilis*, antimicrobial activity was observed in one venom with average zone of inhibition diameters from 0.77-1.20 cm for concentrations 100-1000 μg/mL. Future directions include repeating and completing additional Kirby-Bauer assays, performing a minimum inhibitory concentration

(MIC) assay, and characterization of the active components in venoms exhibiting antimicrobial properties.

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DEDICATION

This work, like the rest of it, is dedicated to my parents, grandparents, and loving partner for their continuous support in my adventure to craft a future that fulfills all my dreams.

Cheers.

CHAPTER ONE

Introduction

The sheer number of surfaces we touch each day from the moment we awake to the moment we drift off to sleep is remarkable. The door handle on the way into work, the front desk pen at your doctor appointment, the pump you used to fill up your car with gas, knobs and toilet handles with every visit to the restroom. The exchange of material with each touch is simple but critical; a mixing of the slew of microorganisms that were once sitting on your hand and the surface just prior. This transmission is harmless except in the case of disease-causing, pathogenic microbes, particularly pathogenic bacteria that multiply just sitting on the surface. Any surviving pathogenic bacteria making it to the surface of your skin could be further introduced into your system when you rub your nose, eat without washing your hands, or otherwise introduce the bacteria past the body's outer protective skin layer. If the pathogen is introduced inside the body at satisfactory amounts to withstand the initial immune response, the pathogen will then do as a pathogen does – divide exponentially, infect the host, and cause disease.

The molecular fight against these crafty microorganisms accidentally began in 1928 when Alexander Fleming discovered the first antibiotic during his studies of *Staphylococcus aureus*.[1,2] Famously, Fleming had left a petri dish smeared with *S. aureus* out on his laboratory bench when he left for vacation. Upon his return, he noted a mold that had contaminated his dish, but peculiarly observed that the mold inhibited the growth of surrounding bacteria. Fleming investigated the contaminant and later discovered the antibacterial agent known as penicillin.[1] The structure of penicillin was determined in 1949 by X-ray crystallographer Dorothy Crowfoot

Hodgkin, and the drug later became available in the United States during the 1950's for over-the-counter use.[3] For almost ten years, penicillin was widely available without prescription and used irresponsibly by the public; however, it was not the only antibiotic following such a pattern.[3] The 1940-1960 'Golden Age' of antibiotic discovery grew to include other major classes of antibiotics such as tetracyclines, vancomycin, and ampicillin.[4] This time period is said to have transformed clinical medicine by providing effective cures to some of the most prevalent diseases of the time, including agricultural disease with improvements made in animal health as well.[4,5] However, after the discovery and wide use of these drugs, resistant bacterial strains capable of inactivating the harnessed antibiotic mechanisms became prevalent.[3] In fact, the 'core' population of antibiotic-resistant strains is believed to have been established by the early 1960's within most industrialized countries, and subsequent transmission of these plasmid-encoded resistance mechanisms has contributed to the international propagation of resistance.[3]

Antibiotic resistance is an ever-worsening problem

Since the "Golden Age," many newer antibiotics have been produced synthetically by chemical modification of pre-existing antibiotics to maximize the efficacy and broad spectrum activity in subsequent drug generations.[5] Yet even with new research and discovery, antibiotic resistance has continued to become a pressing worldwide problem.[6] To survive the effects of antibiotics, germs are constantly finding new defense strategies, called resistance mechanisms, to defeat the drugs designed to kill them.[7] Germs that successfully outsmart the antibiotic will not be killed, therefore enabling successive resistant generations through replication. Resistance, on the other hand, may also pass directly between germs spreading into new settings and countries that may have never encountered the initial antibiotic.[8] This transmission is made possible by

way of mobile genetic elements including plasmids, transposons, and bacteriophages.[8] Bacteriophages, or viruses that attack bacteria, transfer resistance by transduction, which is the act of transferring deoxyribonucleic acid (DNA) from one bacteria to another.[8] Additionally, genes that confer antibiotic resistance can be picked up, either via plasmid or transposon, by nearby bacteria or directly transferred between bacteria via conjugation.[8] To convey the pervasiveness of these operations, experiments on rate of conjugation suggest that frequency of transmission in nature is several orders of magnitude greater than laboratory conditions allow.[3]

As shown, the bacterial environment helps in many ways to escalate resistance, but the ways in which bacteria activate their own internal resistance mechanisms have yet to be discussed. Resistance mechanisms are constantly evolving, thereby requiring the scientific community to stay on their toes as they attempt to understand each new identified threat. The demand placed on the scientific community is further straining with no current systematic surveillance of antibiotic resistance across nations.[6] The current deficit in international resistance data, however, has not inhibited our understanding entirely. Resistance mechanisms generally entail bacteria limiting or evading the antibiotic effects. Some of the better understood mechanisms include bacteria restricting the access of the antibiotic by either changing or limiting the number or entryways for the drug, or using pumps in the cell wall to remove whatever amount of drug that had entered.[8] A direct defense strategy of bacteria is to change or destroy the antibiotic with enzymes, while another indirect strategy could include bypassing the antibiotic effects by altering targets of the antibiotic to no longer associate with the drug.[8] Nevertheless, the complexities associated with the emergence and passing of resistance cannot be overemphasized.[3]

The successful use of any antibiotic is compromised by the potential development of tolerance or resistance to that compound from the time it is first administered.[3] Since introduction, millions of metric tons of antibiotics have been produced and employed for a wide variety of purposes, and the decrease in cost associated with this mass production has encouraged many nonprescription and off-label uses.[3] Antibiotics are among the most commonly prescribed drugs used in human medicine; however, up to 50% of all antibiotics prescribed for people are either not needed or not optimally effective as prescribed.[6] Wide administration of these drugs given to people, animals and crops together have encouraged development of resistance by increasing the rate and spread of each 'first administration.' Comparison between the amount of antibiotics used in humans versus animals for food suggest antibiotic use is higher in food production.[6] For humans, antibiotics are often administered through intravenous (IV), intramuscular (IM), or oral routes depending on the medical application.[7] In animals, antibiotics can be administered topically or by placement into feed and/or water for both treatment and/or prevention means.[7] Crops are commonly sprayed by truck or airplane with a mixture of pesticides containing antibiotics and fungicides to prevent or treat plant diseases.[7]

With such broad use, resistance could effectively develop in a crop and find its way into both animal and human environments alike to spread. As an example of possible spread, three farms in South Africa were followed to determine the antibiotic resistance patterns of *Escherichia coli* and *Staphylococcus aureus* from the raw meat and feces of game species within the farms.[9] Results highlighted the importance of hygiene and food safety with evidence of antibiotic resistant bacteria cross-contaminating the meat during slaughter by way of personnel handling, equipment and carcass fecal matter.[9] Although it seems unlikely that antibiotic resistant bacteria would be found in undisturbed wildlife, the movement of antibiotic resistant

genes can reach even isolated locations from pollution of human and farm animal environments.[9] The development and overall distribution of antibiotic resistance throughout the biosphere are the result of many years of selection pressure from human applications via underuse, overuse, and misuse combined.[3] According to some, there is perhaps no better example of the Darwinian principles of selection and survival.[3] Yet this evolutionary process is intensified with anthropogenic influence superimposed on nature, which has brought the now product of natural selection to the forefront against us. Efforts to slow this progression have been difficult to implement on a global scale, which has resulted in the evolution of many bacterial pathogens into multidrug resistant forms after repeated antibiotic misuse.[3]

Antibiotic resistance is not only complex in itself but elicits complex outcomes for all affected. Although any person at any stage of life can be a victim of antibiotic resistance, the most vulnerable – the young, elderly, and sick – are often disproportionately affected as they receive medical care.[7] Hospital transmission is therefore the most deadly form of infection, but community spillover becomes much harder to control.[7] Antibiotic resistant infections threaten modern medicine, and with decreasingly effective treatments available many necessary life-saving medical advances become a serious risk. According to 2019 report from the Centers for Disease Control and Prevention on Antibiotic Resistance Threats in the United States, antibiotics are annually necessary for the minimum 1.7 million adults who develop sepsis, 1.2 million women undergoing cesarian sections, 30 million individuals at risk of infection due to chronic diabetes, 33 thousand people receiving organ transplants, 650 thousand people requiring outpatient chemotherapy, and others. These applications rely on antibiotics to treat infections that patients are particularly vulnerable to, and increasing antibiotic resistant infection only brings about additional illness, stress, cost, and even death to the already suffering patient outcomes.[6]

Put simply, we are completely dependent on slowly failing antibiotic technology, and there are no simple solutions.[3]

The first report documenting the threats of antibiotic resistance in the United States from the Centers for Disease Control and Prevention was published in 2013, and the only subsequent report to date was released in 2019. In 2013, there were more than 2.6 million antibiotic resistant infections and nearly 44 thousand deaths.[7] After the release of these numbers, prevention efforts began mainly in hospitals, and some communities, to minimize spread. Challenges in lowering hospital transmission were focused on healthcare environmental spread, such as from bedrails, devices, and other high-touch surfaces.[7] Community spread also brought its own challenges of general poor hygiene, spread into food supply, and inconsistent use of safe sex practices.[7] The 2019 update report revealed 35 thousand deaths due to antibiotic resistance, showcasing an 18% decrease in mortality rate and suggesting that prevention efforts have been effective in some ways.[7] Yet, the number of overall infections remained high with 2.8 million individuals affected by antibiotic resistance in 2019.[7]

Regarding the global-scale, experts relay sobering forecasts for mortality rates of antibiotic resistant infection, indicating that the current 700 thousand a year will evolve into an annual 10 million by 2050.[10] Without an alternative, antibiotic resistance will become the main cause of death in less than 30 years. Additional warnings regard the economic costs of this trend. The previously estimated economic cost of resistance to the United States in 2013 was as high as $20 billion in direct healthcare costs, with additional costs to society as high as $35 billion in lost productivity.[6] When compared to the 2050 projected global cost of $100-210 trillion, serious concerns come about for low-income countries and global poverty rates.[11] Antibiotic resistance could effectively take over health, decrease global productivity and trade, devastate livestock

output, and more with under-privileged countries being most affected.[11] The burden of antibiotic resistant disease is already present in countries like India, where a large portion of the population is immunocompromised on account of diabetes, renal failure, heart diseases, cancer, and HIV enabling higher infection rates.[4] Therefore, the development of alternative therapeutics is urgent, as it is proven bacteria will inevitably find ways of resisting the antibiotics we develop.[4,6]

Snake venoms as an alternative to antibiotics

Despite the advances made in antibiotic resistance prevention, the difficulty is identifying new mechanisms to kill multidrug resistant bacterial pathogens has been described as depressing.[4] Many experts, including the CDC, believe the world has already stepped into a 'post-antibiotic' era, meaning untreatable infection is not a far off threat, but the current reality and set only to get worse.[7] Among all resistant bacteria, gram-negative pathogens are particularly dangerous as they are notably becoming resistant to nearly all antibiotics, leading to high morbidity and mortality of affected individuals.[6,12] To a lesser extent, some gram-positive infections, such as those caused by *Staphylococcus spp.* and *Enterococcus spp.*, are also instigating some worry in experts.[6] Many researchers have looked back to nature for alternative therapeutics, as nature has been the source and inspiration for drug discovery for thousands of years.[4] Many potential drug targets remain to be exploited in antimicrobial study, and venom has piqued the interest of many experts as a rich source of bioactive molecules.[3,4] A wide variety of predatory and/or parasitic animals including snakes, scorpions, spiders, conus and wasps have been studied and reported to produce antibacterial properties within their venom.[13] As an example, Iranian researchers investigated the *in vitro* antibacterial effects of the *Vespa orientalis* wasp and found that crude venom inhibited the growth of two gram-positive bacteria:

Staphylococcus aureus and *Bacillus subtilis*, and two gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumonia* with more significant inhibition sensitivity in the gram-positive bacteria.[13]

William (Bill) Edward Haast is a notable pioneer of venom production having founded Miami Serpentarium Laboratories in 1946 to produce high-quality snake venom suitable for use in worldwide venom research.[14] Haast believed in the medicinal properties of venom, and was known to inject a cocktail of venoms from 32 lizards and snakes on a daily basis.[15] Living to be 100 years old while remaining fit with an unusually youthful appearance, he called himself the "poster boy for the benefits of venom," as he believed the secret to his longevity came from such careful doses.[15] Haast originated the concept of using venoms to cure polio, treat arthritis and multiple sclerosis, and his self-immunizations against snakebites beginning in 1948 enabled his blood to save the lives of 21 snakebite victims from around the world.[14,15] While some of this may seem bizarre, Bill Haast opened the door for many researchers to consider the potential power of snake venom. The first successful drug developed based on snake venom components was Captopril®, created in 1975 from the venom of the Brazilian arrowhead viper (*Bothrops jararaca*), and used to treat cardiovascular disease and high blood pressure after FDA approval in 1981.[16] Since this development, snake venoms have become an important natural supply of bioactive molecules for the development of new drugs.[16]

Snakes have arguably been among the most despised creatures within the animal kingdom, representing both harm and evil, but can elicit a mixture of reactions including fear and fascination.[10] On the other hand, snakes play an important part in the ancient Greek world with Asclepius, the God of Medicine, having been depicted holding a stick entwined with a snake, still used today to symbolize medicine and pharmacy.[4] This dual embodiment of good and evil

has not only carried on with the snakes image, but unexpectedly become supported by increasing evidence showing snake venom compounds can help save lives rather than end them.[10] Currently, a handful of snake venom based drugs exist on the market to target various cardiovascular disorders and blood abnormalities, but other applications, such as the antibiotic potential, are under investigation.[10] Peculiarly, snakebite victims have a remarkably low incidence of microbial infection at the wound site even with the heavy colonization of pathogenic bacteria found in the mouth of the snake.[4,10] This observation sensibly suggests the presence of antimicrobial compounds within the injected venom, and it has been hypothesized that these components of the venom were selected under evolutionary pressure to protect the snake from microorganisms present on their prey.[4]

Methods used to characterize antimicrobial activity of snake venoms

Snake venoms are complex mixtures containing hundreds to thousands of rare bioactive peptides and toxins, constituting up to about 90-95% of the dry weight of the venom.[4,16] Snakes use their venom primarily to incapacitate and immobilize their prey, secondarily as a defensive tool against predators, and tertiarily as a digestion aid.[4] However, the vast source of peptides produced have not been thoroughly explored for their antibiotic potency, and with so many venoms to examine, the number of drug leads derived from snake venom components can only increase in the future.[4,10,16] Many studies today investigate the antibiotic potential of snake venoms using the Kirby-Bauer (KB) disk diffusion assay and other quantifying assays such as the minimum inhibitory concentration (MIC) assay. For example, researchers analyzing the venom of the Iranian Viper (*Vipera latifii*) used the KB assay to illustrate inhibitory effects of the crude venom against *B. subtilis* and *S. aureus*, and subsequently used the MIC assay to

demonstrate that at some concentrations the venom was more effect than standard tetracycline antibiotics.[17]

The disk diffusion method was originally published by W. M. M. Kirby and colleagues at the University of Washington School of Medicine in 1956; however, standardization of the method occurred later in 1961 by the World Health Organization (WHO) after Kirby and his colleague, A. W. Bauer, published updates to previous findings.[18] The result of WHO standardization was the KB assay, named after Kirby and Bauer, which provided a more efficient way to determine the susceptibility of bacteria to various antimicrobials.[18] The KB assay is commonly employed in both medical and research laboratories against pathogenic aerobic and facultative anaerobic bacteria to either assist a physician in selecting treatment options for their patients, or clue a scientist into bacterial susceptibility to different compounds. In a medical setting, a physician cares only about the determination of susceptibility or resistance, while in a research setting, the measured inhibition zone size plays an important role.[18] For the KB assay, a pathogenic organism is grown on Mueller-Hinton agar in the presence of compound-impregnated filter paper disks, and the presence or absence of growth around the disks after incubation provides an indirect measure of the ability of the compound to inhibit the bacterial organism.[18] For positive results, the area of absent growth around the disk is called a zone of inhibition, and comparison of this measurement with other zones can help determine potential antibiotic power.

For this investigation, the KB assay was used to analyze the antimicrobial potential of six venoms of diverse origin against *Bacillus cereus*, *B. subtilis*, *E. coli* and *Proteus vulgaris*. Equal representation of gram-positive and gram-negative bacteria was important for the design of this study and continued analysis. *B. cereus* is a facultatively anaerobic, gram-positive bacterium that is frequently found in soil, vegetation, and contaminated food.[19] It commonly causes intestinal

illnesses with nausea, vomiting, and diarrhea; however, it has been associated with serious infections in immunocompromised hosts.[19] *B. subtilis* is a spore forming, motile, gram-positive, aerobe.[20] Similarly to *B. cereus*, *B. subtilis* is found in soil and vegetation, but can contaminate food causing opportunistic infection in the immunocompromised.[20] *E. coli* is a facultatively anaerobic gram-negative bacterium that is known to be part of normal intestinal flora, but can also be the cause of many diarrheal illnesses.[21] *P. vulgaris* is a motile, aerobic, gram-negative bacterium known for its swarming activity, common presence in the fecal flora of humans, and typical prevalence in urinary tract infections.[22] Following the trend seen by many other researchers, it is reasonably presumed that gram-positive bacteria would display higher susceptibility to antimicrobial compounds than gram-negative bacteria.

The six featured venoms in this study originated from the Western Diamondback Rattlesnake (*Crotalus atrox*), Eastern Cottonmouth (*Agkistrodon piscivorous pisxivorous*), Rinkhals (*Hemachatus haemachatus*), Russell's Viper (*Vipera russelii*), Monocled Cobra (*Naja naja kaouthia*), and Common Night Adder (*Causus rhombeatus*) in no particular order. Due to the ongoing nature of this experimentation, all St. Mary's University researchers remain blinded to the venom-correlated results; however, a brief background of each snake's geographic location and venom can be discussed. Western Diamondback Rattlesnakes are commonly found across the southern United States, but also extend well into central Mexico.[23] These snakes are particularly aggressive and easily excitable, and their venom causes more fatalities than any other snake in the United States.[23] The Eastern Cottonmouth, also known as the water moccasin, is found mainly in southeastern United States.[24] Although these snakes are thought to be aggressive and inflict invariably fatal outcomes, Eastern Cottonmouths typically only bite out of defense and would prefer to save their fatal venom for prey.[24] The Rinkhals is concentrated in

Southern Africa, and its cytotoxic venom causes pain and tissue damage to the affected.[25] Snake bites and fatalities though are both extremely rare, yet the Rinkhals can spit its venom up to 3m ahead at its prey.[25] According to some, the Russell's Viper is one of Southeast Asia's most dangerous snakes, whose venom can elicit kidney, heart, and/or respiratory failure within 24 hours of an attack.[26] Similarly across South and Southeast Asia is the Monocled Cobra, responsible for causing rate of fatality due to snake venom poisoning in Thailand.[27] Last but not least, the Common Night Adder is found in sub-Saharan Africa, and the cytotoxic venom of this snake requires some victims, especially children, to be hospitalized.[28]

CHAPTER TWO

Materials and Methods

Six venoms were harvested from snakes of diverse origin, as previously explained, and aliquots of each were frozen down. Aliquots were kindly provided by our collaborator at The University of Texas Health Science Center (UTHSC) and kept frozen at -20^{o}C until use. The four tested bacteria: *B. cereus, B. subtilis, E. coli* and *P. vulgaris*, were grown in the 37^{o}C incubator overnight on Luria-Bertani (LB) agar plates and kept in the 4^{o}C fridge for the duration of the experiment. Beginning each KB assay, a Bunsen burner was lit inside the bench space and allowed to burn for 5 minutes prior to start to sterilize the air and working space. Diffusion disks were inoculated with crude venom in concentrations ranging from 0.1-1000 μg/mL and allowed to dry for at least one hour in the refrigerator, although drying times varied up to 24 hours. Using an inoculating loop, 2-3 colonies were taken from bacterial subcultures and suspended in 5 mL of Luria-Bertani (LB) broth. Using a micropipette, 50 μL of the inoculated broth were delivered to Mueller-Hinton agar plates and spread evenly using a bacterial spreader. Plates were allowed to dry upside down for 5-10 minutes prior to disk placement. The dried inoculated disks were placed on Mueller-Hinton agar in ascending concentration along with negative control disks and positive antibiotic controls. Placement of the disks was performed with tweezers sterilized in 70% ethanol before and between disk types. The KB assay plate was placed in the 37^{o}C incubator overnight to allow the assay to run its course, and analysis occurred the following day.

Positive results showed a zone of inhibition around the diffusion disc, and negative results displayed the absence of a zone of inhibition. Measurements of inhibition zones were taken in centimeters (cm) along the zone diameter three ways, averaged, and recorded. If a zone

diameter could not be symmetrically measured, the representative radius was measured and doubled prior to measurement documentation. Images of all plates were taken on an Analytik Jena UVP UVsolo Touch imaging system to correlate with recorded measurements. Data were analyzed for antibiotic potential based on the correlation of results seen in replicates and other tested concentrations. Randomly identified zones of inhibition were determined to be rather insignificant upon this initial experimentation, and the rationale for this is explained in chapter 4.

CHAPTER THREE

Results

KB assay results for *B. cereus* against all venom samples were negative. One random zone of inhibition was observed for venom 3 at 5 μg/mL, but a zone of inhibition was not observed in the other two replicates for the experiment. These zones and the corresponding negative result will be explained further in chapter 4. Measurements from the *B. cereus* KB assays may be viewed in Table 1. Similarly, results for *E. coli* against all venom samples were negative but included more frequent observation of random zones. This includes one random zone against venom 3 at 10 μg/mL, one random zone against venom 6 at 0.1 μg/mL, and two random zones against venom 5 at 100 μg/mL. No other zones outside of these replicates were observed. Measurements for the *E. coli* KB assays may be viewed in Table 2. Due to unspecified reasons, experiments for *P. vulgaris* were only performed with venoms 1-3, and for those tested, *P. vulgaris* KB assays showed negative results with individually random zones of inhibition detected against venoms 1 and 2 both at 0.1 μg/mL. Measurements for the *P. vulgaris* KB assays are available in Table 3.

Table 1. Kirby-Bauer Assay Measurements for *B. cereus*. All measurements are given in centimeters. Average measurements of observed zones of inhibition are available in the shaded section below with an asterisk (*) indicating the number of replicates represented in the average. All positive control measurements are for a single replicate of the indicated ampicillin (Amp), tetracycline (Tetra), and vancomycin (Vanc) antibiotics.

Venom #	Neg Ctrl	0.1 µg/mL	1 µg/mL	5 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	Positive Controls
1	-	-	-	-	-	-	-	-		**Amp: 0.92**
2	-	-	-	-	-	-	-	-		**Tetra: 1.68**
										Vanc: 1.60
3	-	-	-	***1.07**	-	-	-	-		**Amp: 0**
4	-	-	-	-	-	-	-	-	**N/A**	**Tetra: 1.53**
										Vanc: 1.50
5	-	-	-	-	-	-	-	-		**Amp: 0**
6	-	-	-	-	-	-	-	-		**Tetra: 1.77**
										Vanc: 1.60

Table 2. Kirby-Bauer Assay Measurements for *E. coli*. All measurements are given in centimeters. Average measurements of observed zones of inhibition are available in the shaded section below with an asterisk (*) indicating the number of replicates represented in the average. All positive control measurements are for a single replicate of the indicated ampicillin (Amp), and vancomycin (Vanc) antibiotics.

Venom #	Neg Ctrl	0.1 µg/mL	1 µg/mL	5 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	Positive Controls
1	-	-	-	-	-	-	-	-	N/A	Amp: 1.85 Vanc: 0
2	-	-	-	-	-	-	-	-		
3	-	-	-	-	*0.96	-	-	-		
4	-	-	-	-	-	-	-	-		Amp: 1.99 Vanc: 0
5	-	-	-	-	-	-	**0.83	-		
6	-	*0.79	-	-	-	-	-	-		

Table 3. Kirby-Bauer Assay Measurements for *P. vulgaris*. All measurements are given in centimeters. Average measurements of observed zones of inhibition are available in the shaded section below with an asterisk (*) indicating the number of replicates represented in the average. All positive control measurements are for a single replicate of the indicated chloramphenicol (Chlor), and tetracycline (Tetra) antibiotics.

Venom #	Neg Ctrl	0.1 µg/mL	1 µg/mL	5 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	Positive Controls
1	-	*1.18	-	-	-	-	-	-	N/A	Chlor: 4.25 Tetra: 1.02
2	-	*1.20	-	-	-	-	-	-		
3	-	-	-	-	-	-	-	-		

Assays with *B. subtilis* against venoms 1-4 and 6 were all negative with one random zone of inhibition recorded against venom 2 at 50 μg/mL. Positive results were seen in *B. subtilis* KB assays against venom 5. Results showed one random zone with venom 5 at 10 μg/mL. Arguably non-random zones, however, were observed across the KB assay runs for venom 5 at 100 μg/mL in two replicates, and both 500 μg/mL and 1000 μg/mL in all three replicates. The average diameter for zones of inhibition seen at 100 μg/mL, 500 μg/mL, and 1000 μg/mL were 1.05 cm, 1.20 cm, and 0.77 cm, respectively. Explanations for the differences observed in identical concentrations between repeat experiments with *B. subtilis* and venom 5 will be discussed further in chapter 4. Measurements for these KB assay results are seen in Table 4. Images of both KB experiments completed with *B. subtilis* against venom 5 may be viewed in Figures 1 and 2.

Table 4. Kirby-Bauer Assay Measurements for *B. subtilis*. All measurements are given in centimeters. Average measurements of observed zones of inhibition are available in the shaded section below with an asterisk (*) indicating the number of replicates represented in the average. All positive control measurements are for a single replicate of the indicated ampicillin (Amp), tetracycline (Tetra), and vancomycin (Vanc) antibiotics.

Venom #	Neg Ctrl	0.1 µg/mL	1 µg/mL	5 µg/mL	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1000 µg/mL	Positive Controls
1	-	-	-	-	-	-	-	-	N/A	Amp: 2.95 Tetra: 3.48 Vanc: 2.28
2	-	-	-	-	-	*0.98	-	-		
3	-	-	-	-	-	-	-	-		
4	-	-	-	-	-	-	-	-	N/A	Amp: 3.15 Vanc: 2.34 Chlor: 2.67
5a	-	-	-	-	-	-	-	-	***0.77	
5b	-	-	-	-	*1.14	-	**1.05	***1.20	N/A	Amp: 3.24 Vanc: 2.49 Chlor: 2.96
6	-	-	-	-	-	-	-	-		

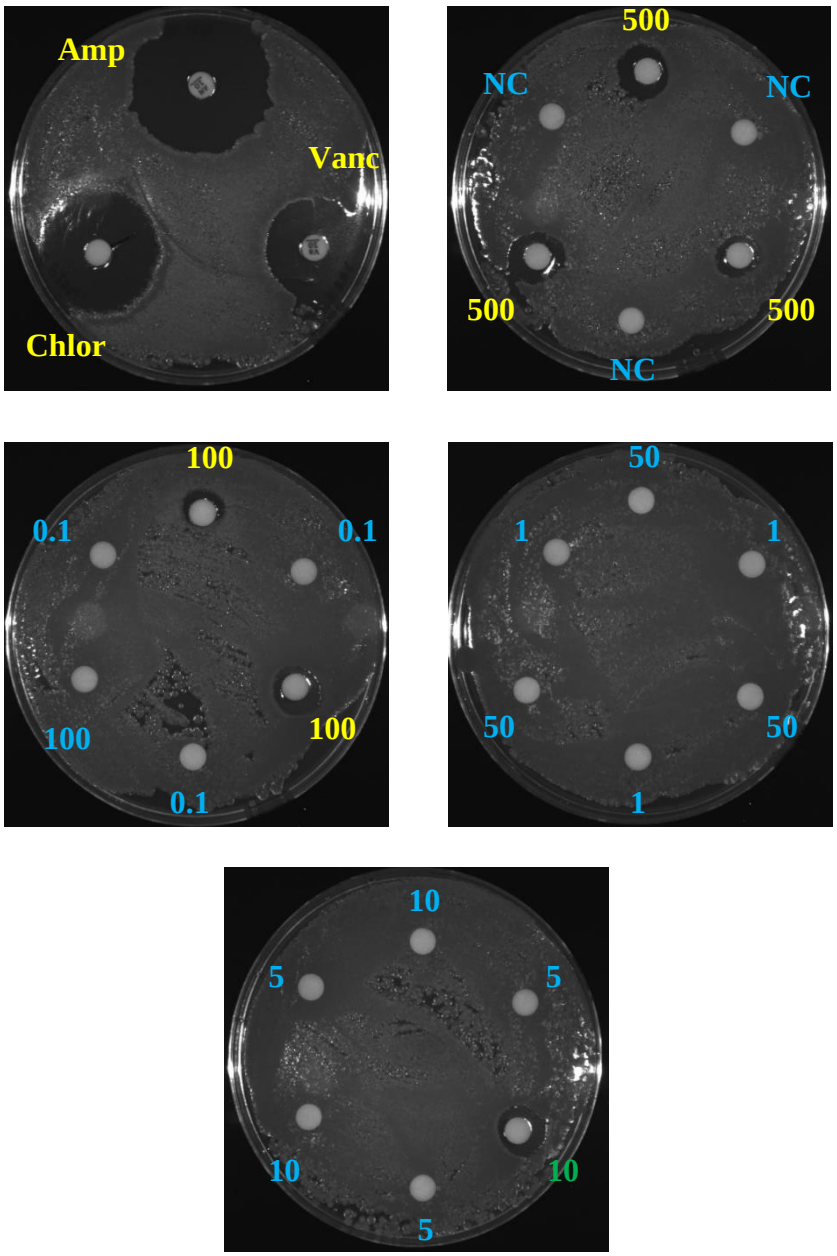

Figure 1. Positive Kirby-Bauer Assay Plate Images with *B. subtilis* against venom 5 (Experiment 5b). Plate images are shown with yellow disk identifiers indicating a positive zone of inhibition, green disk identifiers indicating an arguably random zone of inhibition, and blue disk identifiers indicating a negative result or the absence of a zone of inhibition. Number values given are representative of the venom concentration present in ug/mL. The positive controls are ampicillin (Amp), vancomycin (Vanc), and chloramphenicol (Chlor).

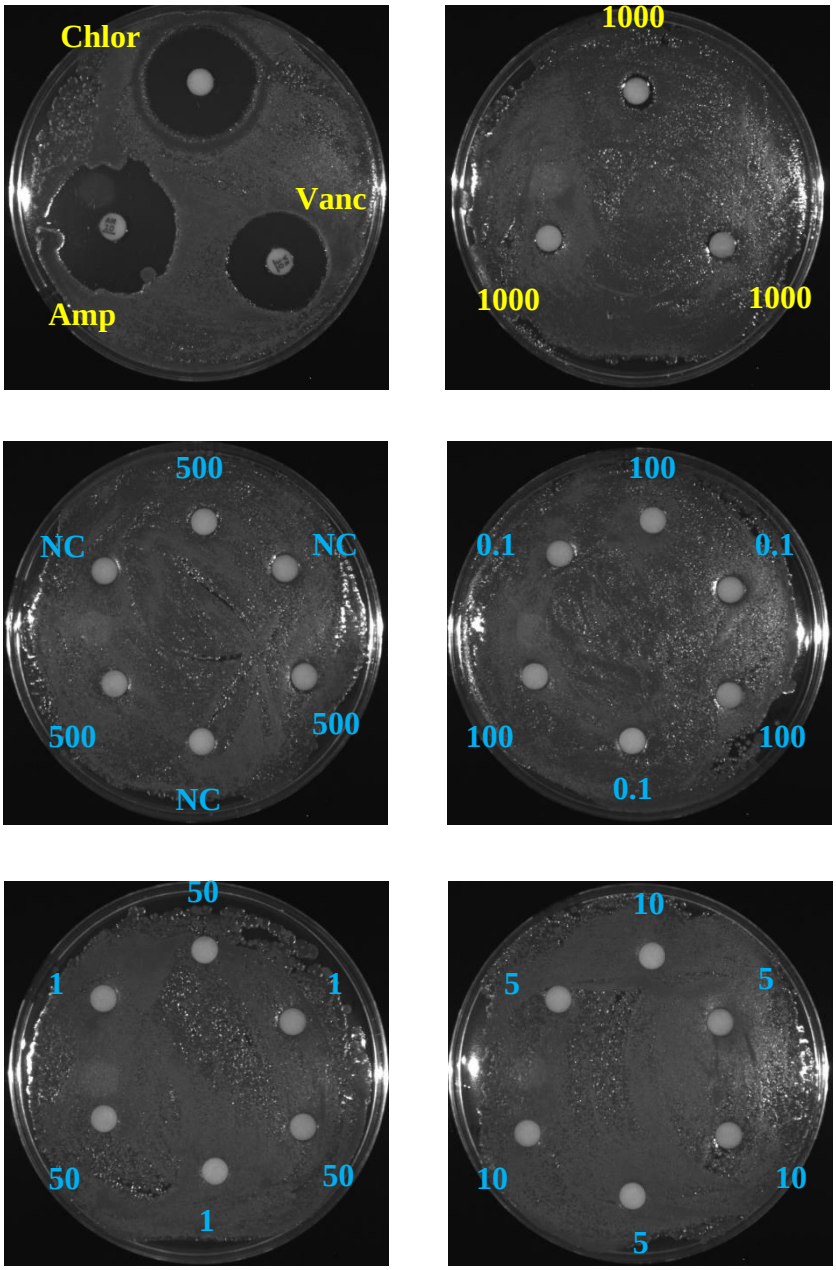

Figure 2. Positive Kirby-Bauer Assay Plate Images with *B. subtilis* against venom 5 (Experiment 5a). Plate images are shown with yellow disk identifiers indicating a positive zone of inhibition and blue disk identifiers indicating a negative result or the absence of a zone of inhibition. Number values given are representative of the venom concentration present in ug/mL. The positive controls are ampicillin (Amp), vancomycin (Vanc), and chloramphenicol (Chlor).

CHAPTER FOUR

Discussion

The present investigation describes the assessment of antibiotic capability using six venoms from diverse origin against four bacteria known commonly to cause opportunistic infection. Crude venom 5 KB assays exhibited notable antibiotic proficiency against *B. subtilis* in all or multiple replicates at concentrations of 100 μg/mL, 500 μg/mL, and 1000 μg/mL with zone sizes ranging from 0.77-1.20 cm; although, the differences in the size of produced zones of inhibition between corresponding concentrations of repeat experiments require explanation. Regarding the differences between measurement values in Table 4 and observed zones in Figures 1 and 2, one must remember that *B. subtilis* is a motile bacterium, meaning that it can move on top of agar that it grows upon. It is certain that overnight incubation periods between the successive 5a/5b *B. subtilis* KB assays with venom 5 were not identical, meaning that in the subsequent experiment, 5a, the bacterium may have had the opportunity to spread into the inhibition zones before data were recorded, producing the smaller zones. The noted trend from experiments showed an average 1.20 cm zone against venom 5 at 500 μg/mL in all three initial replicates and an average 0.77 cm zone against venom 5 at 1000 μg/mL in all three subsequent replicates, supporting this suggestion. Another possible reason for the variation could be a fault of the disk itself. Researchers similarly using the KB assay to investigate crude snake venom have determined the possibility that the disk itself can act as a filter, preventing complete withdrawal of the venom components into the culture.[17] To lean into this possibility, it is also suggested to keep opened antibiotic susceptibility disk cartridges in a storage container for no more than 1 week, and this time cutoff was surpassed in occasional experimentation.[18]

Reasoning for false positive determination of documented random zones of inhibition within *B. cereus*, *P. vulgaris*, *E. coli* and *B. subtilis* may also be elucidated, but come with a wide array of possible explanations for the observation. The largest reason for false positive determination was the lack of representation in multiple corresponding concentrations and replicates. It is possible that ethanol residue could have been left on the tweezers used to transfer diffusion disks, or that fungal spores in the air could have contaminated the diffusion disk itself or Mueller-Hinton agar with which the disk was placed on. Either of these possibilities could produce false positive results, as both ethanol and fungi can minimize and eliminate bacterial proliferation. A perhaps more feasible possibility that may explain the prevalence of false positives is based upon the depth of Mueller-Hinton agar. In plates that are too shallow, the antimicrobial compound will diffuse further than it should, as it diffuses in three dimensions, therefore producing a larger zone of inhibition rather than a deeper layer.[18] On the other hand, plates poured to depths of >4mm could produce false negative results with too deep of a layer to diffuse into.[18] With this in mind, it is important to note that all plates used in the experiment were hand poured, meaning there was likely variability in the depth of agar.

A potential systematic error is present with literature suggesting that bacterial organisms must be in the log phase of growth for results of KB assays to be valid, and it is therefore recommended that subcultures of the organisms for testing be made the previous day.[18] In experimentation, subcultures were irregularly prepared. Both *B. subtilis* and *B. cereus* were completed with subcultures made the day prior to each assay, but *E. coli* and especially *P. vulgaris* were not. This could be another explanation for the higher prevalence of random inhibition zones seen in both *E. coli* and *P. vulgaris*. Overall, overnight incubation times for subcultures and KB assays were not standard, likely playing into the variation seen in results.

Due to this, it is entirely possible that both undergrowth, leading to false negatives, and overgrowth, leading to false positives, could be equally possible as having affected results.

Nevertheless, comparison of these data with that of other researchers does not generate immediate concern over validity. With other researchers alike, applied snake venoms showed to be more effective on gram-positive bacteria, such as *B. subtilis*, than gram-negative counterparts.[13,17,29] As discussed, snake venom is comprised of bountiful proteins and peptides with uncharacterized bioactive capability, so it is not a surprise that over 58% of studies focused on antibacterial properties report effective results.[30] Previously mentioned researchers investigating wasp venom correspondingly indicated that *B. subtilis* was the most sensitive bacterium to their crude wasp venom, which is echoed in this report here with snake venom.[13] Venom compositions of snakes and wasps, however, are likely very different regardless of similar results, as venoms between snake families are indeed known to significantly differ in composition proportions.[30]

With ongoing experimentation, future directions are extensive with the most pressing being to complete the *P. vulgaris* KB assays against the remaining venoms 4-6. This is especially important considering venom 5 was the compound showing antimicrobial capability. As an extension of these assays, the *B. subtilis* KB assays could be repeated with strict standard incubation times, completion of subculture from fresh *B. subtilis* prior to every assay, and care not to reuse freeze-thawed venom aliquots. This would help enlighten the hypothesis that differences in subsequent venom 5 KB assays were due to lack of standardization rather than other reasons. A change in diffusion disk inoculation could be an additional variable to try, as researchers similarly investigating snake venom using KB assays chose to impregnate the disk once it had already been placed on the culture-spread plate, which prevents any loss of venom

that could dry to another surface.[17] Furthermore, antibacterial testing of snake venom currently lacks a major push towards quantification of inhibition, indicating that MIC assays would be the logical next step after KB assay completion for venom 5 and any other potentially interesting compounds.[30] Supplementary next steps could be acquiring more bacteria and/or snake venoms to test, of course, or using more traditional antibiotics in testing to actively compare the efficacy of snake venoms against positive data. These changes in particular would likely lead to similarly diverse outcomes, as researchers have identified that certain snake families may be better at targeting various bacterial classes than others.[30]

This experiment, like many others, tested crude venoms. Therefore, additional studies would need to be completed to determine the exact components within venom 5, and pending others, that would be developed as potential antimicrobial drugs. After all, the zone sizes observed in KB assays can only tell so much, as it is through *in vivo* testing that characterization and clarity come regarding a compound's true ability to resolve infection.[18] Nevertheless, humanity is still losing the race against antibiotic resistance, and the problem will only get worse if we do not concertedly act now. Unfortunately, as reinforced by the COVID-19 pandemic, decisive actions that require significant commitment and enforcement are never popular, even in the event that lives could be saved.[3] New antimicrobials will always be necessary to fight against pathogenic microorganisms because once each new antibiotic compound is released, the countdown starts for how long it will be effective.[4,7] While the development of new treatment forms is a key action moving forward, this must be coupled with dedicated efforts toward preventing infection in the first place, and stopping the global spread of resistance as much as possible.[6]

The Centers for Disease Control and Prevention released recommendations in the 2019 report on Antibiotic Resistance Threats outlining five core actions they anticipate will better prepare the United States for the global impending resistance. These actions were comprehensive infection control, tracking potential threats and data, concerted antibiotic stewardship, investments toward vaccine/therapeutic development, and improving environmental protection.[7] As a nation we must stop playing the blame game because in reality all of humanity has been complicit in the development of this worldwide problem, so the only way out is to assemble together. Antibiotic resistance is not a problem 'over there' in someone else's hospital, state or country, but rather reality right under each one of our noses.[7] Given the increasing knowledge and research going into early detection of potentially resistant microorganisms and development of alternative therapeutics to combat current resistance, it is mandatory that the world takes advantage of these new understandings and technologies to make a change.[3] If not, the post-antibiotic era awaits our sorry descent.

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