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FINE FOCUS

AN INTERNATIONAL MICROBIOLOGY JOURNAL FOR UNDERGRADUATE RESEARCH

MISSION

We publish original research by undergraduate students in microbiology. This includes works in all microbiological specialties and microbiology education.

SCOPE

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FINE FOCUS – CATALYZING STEM OPPORTUNITIES FOR UNDERGRADUATES

This fall, our Fine Focus class will be engaged in a very different set of activities compared to recent semesters. The essence of this project is to use the platform of Fine Focus for STEM outreach, in order to allow academically underrepresented students of color, women, and those of lower socioeconomic standing, to be the authors of, and authorities on, their own education. Undergraduate students from the University of Detroit Mercy (UDM) will be provided with appropriate tools to actively participate in scientific writing and the double-blind scientific peer-review process by developing and promoting a digital and print bound journal showcasing research of undergraduates in microbiology internationally. Through this professional development outreach, undergraduate students and their mentors at UDM will also be asked to tell their individual stories on how each overcame specific challenges to pursue a higher education in the sciences, which will be woven into a short film suitable as a recruiting tool for UDM and Ball State. This product, along with a signature issue of Fine Focus, will showcase UDM student achievement in the molecular biosciences while providing these participants the chance to elevate the profile and mission of *Fine Focus* for future student editors and authors.

As the faculty mentor for the *Fine Focus* class at Ball State University (BSU), and Managing Editor of the journal, my learning outcomes for this expanded (15 credit-hour) product-based class will be to cultivate a long-term partnership with UDM faculty and administrators using this opportunity as a precedent. I envision being able to involve UDM students in various future aspects of *Fine Focus* evolution. These partner undergraduates involved in this *Fine Focus* STEM outreach will come away with a working knowledge of how to effectively critique scientific writing and appropriately communicate their assessments to the corresponding authors of all submissions – as well as becoming well-versed in formal communications with invited reviewers from our existing *Fine Focus* Editorial Board. Such "beyond the bench" knowledge is a vital skill in the hard sciences today, when service to the profession is more important than ever before. These soft skills involve not only knowledge, but beneficence, ethics in science, and rigid attention to detail. Such skills are not formally taught in any traditional curricula, and yet eventually expected of all professionals in the sciences; thus, on a very practical level, this STEM outreach endeavor will help to rectify this discrepancy with our target UDM student participants.

However practical and transferable the disciplinary skills described above are for professional development in the sciences, the stories behind each UDM student will be perhaps even more impactful. Learning outcomes for the BSU peer mentors will be shaped primarily by the stories told by each UDM undergraduate and faculty member featured in the short film. The purpose of these stories will be to illustrate the need to involve product-based learning in STEM as early in the undergraduate science curricula as possible, to motivate and inspire students in the midst of shaping their thought life around a career in science. What values fueled each of these UDM students to continue in their pursuit of a higher education? What seemingly insurmountable challenges did some have to overcome (or are still in the process of overcoming) in order to get to this point in their college careers? What advice would each offer to those younger than they? These answers are in and of themselves learning outcomes, however, nontraditional, that will certainly impact our BSU student peer mentors, and a larger audience, through the film we will produce alongside the signature feature issue of *Fine Focus*.

I envision four site visits to UDM during the semester (early-to-mid Sept., Oct., Nov., and Dec.), each 2–4 hours, and each involving a different group of 4–8 BSU peer mentors. These sessions will be designed to be an exchange of ideas. Our side will be to lead the UDM undergraduate participants through the systematic steps of how (and why) *Fine Focus* manuscript management and double-blind scientific peer review functions the way it does. The UDM undergraduate contributions will come in the form of their individual stories. Everyone has a powerful story to tell, and the UDM students represent hidden treasure in terms of what led each of them down a path into the molecular biosciences. Their respective challenges, opportunities, and role models will all play a dominant role in how they will tell their stories to the BSU peer mentors, which will be captured and woven into a short film powerful enough to inspire, recruit, and motivate others in similar circumstances. These stories will capture the unique essence of this experience – STEM outreach to future leaders in science.

BSU student peer mentors involved in development of *Fine Focus*, and UDM undergraduates, will come away with a working knowledge of three fundamental concepts: a) organization of people and resources essential for success of a scientific research journal; b) how to effectively teach and critique scientific writing and communicate their assessments to corresponding authors, a vital skill in the hard sciences, and; c) how to blend their creativity (in overall design and marketing of the journal) with the objective guidelines that govern proper scientific experimental design and strict manuscript format.

How will this happen? Practically speaking, each of the four UDM site visits will be led by a different blend of BSU student peer mentors who will shepherd the target undergraduates through the odyssey of a manuscript upon submission to *Fine Focus* through our "Open Journal System" management system. Step-by-step, these UDM students will be empowered to act on their training, leading eventually to a final decision on one or more *Fine Focus* manuscripts for our signature issue. Additional time will be spent with these students on the urgency of a vibrant marketing team, and the need to evolve continually in order to remain on the edge of our comfort zones and remain innovative.

Student participants will come away with at least several key professional contacts in the fields of microbiology or industry, considering the degree to which each team will need to foster off-campus relationships with practitioners in various disciplines. For example, through regular communication with corresponding authors, members of the external Editorial Board, or the American Society for Microbiology (ASM), students will gain invaluable professional development experience not possible to obtain in traditional content courses.

This level of communication will necessitate each of them to rise to a multitude of varied challenges. For others in the class interested in marketing, contacts will be made in the fields of publishing or the creative arts, during marketing initiatives. Invited speakers we will host at BSU will serve as key resources for community engagement, successful journal management, and science education and pedagogy, all of which are essential for long-term success of *Fine Focus*. Lastly, we will be hosting our inaugural *Fine Focus* Executive Committee meeting. Three individuals plus myself will constitute our first Executive Committee, the task of which will be to develop a long-term vision statement for sustainability of *Fine Focus* into the foreseeable future. One of our confirmed Executive Committee members will be traveling from Finland, and the cost of this and all relevant expenses for the Committee meeting are included in our operating budget. It is quite important for us to have an in-person meeting rather than implementing it using Skype or other similar approach, owing to the sheer amount of material we will have to cover.

Publishing the signature issue of *Fine Focus*, and production of the film of their stories presents this Fall semester with both a technical outcome – the journal issue – along with a very influential and inspirational broader message for young women in STEM, or anyone who may feel compelled to turn their story of difficulty into a trajectory towards a career in the molecular biosciences, an exciting and ever-expanding field of opportunity.

–JLM

THE COLONIZATION AND ESTABLISHMENT OF THE NEONATAL MAMMALIAN MICROBIOME

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ABSTRACT

In current agriculture practices, such as the dairy industry, the use of antibiotics is being discouraged due to the occurrence of antibiotic resistant bacteria. However, antibiotics are used commonly to treat calf diarrhea, which is a serious issue that negatively influences calf health, growth, and development. Recent research highlights the gut microbiota as a potential source to improve the gut health of a calf, which could minimize the antibiotic use. However, limited knowledge is available for the early life gut microbiota and its relationship with calf's performance. It is known that the microbiota has an influence on immune system development, as well as behavioral development, and metabolic development. Further, an atypical microbial population, or a microbial shift, has been linked to autoimmune, anxiety and metabolic disorders. The process of microbial and host interactions starts at birth, suggesting that mammals are initially colonized by microbes immediately following and during birth. Differing modes of delivery, caesarian or vaginal delivery, and possibly the length of time of the birthing process, may determine initial colonization of the infant. Further, the establishment of the microbiota can be influenced by host genetics, diet, and maternal environment. Therefore, this review aims to summarize the current understanding of the neonatal mammalian microbiota obtained from human and mice studies, and to outline future research directions on microbial colonization and possible manipulation strategies that can be used to manipulate the gut microbiota in dairy calves. By understanding the process of how mammals and microbes interact it is possible to better target future research in order to solve the problem of calf diarrhea.

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KEYWORDS

- Early-life Microbiome
- Calf
- Colonization
- Establishment

INTRODUCTION

Calf diarrhea can cause more of a financial loss than any other calf ailment (15). Diarrhea in the young animal prevents absorption of fluids and nutrients, inhibiting growth and health (1). Further, without immediate intervention, calf diarrhea can be transmitted between calves and can result in high mortality. Currently, the common practice to prevent calf diarrhea is the use of antibiotics. Yet, industry is encouraged to reduce the use of preventative antibiotics due to the increasing prevalence of antibiotic resistance. Therefore, an alternative to antibiotic prevention for calf diarrhea is needed. Recent research highlights the gut microbiota as an important factor in immune function development and maintaining neonate gut health, making the gut microbiota a potential source to improve the gut health of calves to reduce the prevalence of diarrhea. However, limited knowledge on the calf gut microbiota is available. Additionally, the mechanisms of how the microbiota influences calf gut development and health are still largely unknown and undefined. Therefore, this review aims to summarize the current understanding of the neonatal mammalian microbiota based on the findings from human and mice studies from three aspects including initial colonization of the neonatal mammals, the impact of the microbiota on gut immune function of the developing neonate mammal, and factors affecting microbial establishment during early life. Further, this review aims to identify the knowledge gap of the calf microbiota in order to direct future research towards identifying potential ways to reduce the incidence of, and possibly prevent, calf diarrhea through the manipulation of gut microbiota.

MAMMALIAN DEVELOPMENT AFFECTED BY MICROBIOTA

It was hypothesized by Louis Pasteur that the microbiota has an important and necessary influence on mammalian life (21). Mammals have a rich and diverse microbial population that interacts with, and influences the development of biological processes in mammals like immune function and metabolic systems (21). Additionally, the microbiota causes long-term impacts on emotional systems (4, 23). The following section of this review will outline specific changes in microbiota and how they can affect the immune system development, anxiety and depression, and digestive and metabolic functions.

IMPACT OF THE MICROBIOTA ON IMMUNE SYSTEM DEVELOPMENT

The interaction between host and microbes is essential for proper immune function development. Young animals enter a critical period soon after birth when exposure to antigens is imperative for immune development. If the exposure is delayed, immune development can be impacted. This can be explained by an experiment using germ free mice (21). Firstly, it is important to note that the authors demonstrated that germ free mice have an atypical cytokine response to orally treated lipopolysaccharide (LPS) when compared to conventional mice. It is important to note that LPS is a large molecule found on the outer membrane of *Gram-negative* bacteria, and the previously

described atypical response differs from a normal response in that the atypical response is delayed and exaggerated. Further, these authors noted that the juvenile mice can resume a normal cytokine response, and behave normally, normal being immediate and moderate, when treated with probiotic *Bifidobacterium infantis*. However, when adult germ free mice were treated with the same probiotics they were unable to be converted back to the normal response. Additionally, these effects on the immune system can have long-term consequences, which are still being investigated (10, 19). It has been found that antibiotic use in early life influenced fecal bacterial composition and can be linked with development of intestinal diseases later in life (17). However, there is convincing research that interruptions in the establishment of the microbiome during early life can result in allergies, asthma and other autoimmune diseases in adults (10). Additionally, it was found that the development of eczema can be minimized if infants were treated with probiotics (10).

After birth, the gut microbiota may contribute to mammalian immune system development through interactions between hosts. The interaction between microbe and host immune development is important, which has been demonstrated by germ free animals as described above. Germ free animals do have immunities; however, they cannot cope with pathogens (22). This reveals that without the proper microbial exposure in early life, the immune system will not properly develop and can increase risk of disease in later life. Further, a more diverse intestinal microbiota during the first week of life is associated with a reduced risk of subsequent eczema in infants (10). Additionally, interventions that enhance microbial diversity in early life may provide an effective means for the prevention of eczema in high risk infants (10).

IMPACT OF MICROBIOTA ON ANXIETY AND DEPRESSION

Initially, the evidence of microbial impact on host behavior was gained from comparisons of germ free and bacterial colonized mice. In order to test the idea that postnatal microbial colonization may affect the development of brain plasticity, researchers compared the hypothalamic-pituitary response to differing levels of stress restraint using genetically identical germ free and specific pathogen free mice (23). In this study, the individual mice were placed into a 50 ml conical tube for 1 hour, or into a glass container lined with ether soaked absorbent paper for 2.5 minutes. The authors found exaggerated stress response in germ free mice when compared to pathogen free mice (23). Further, the same authors observed a reduction in stress response in germ free mice after administration of probiotic *Bifidobacterium infantis*. These results have demonstrated the differences in brain function between germ free and colonized mice, suggesting the potential relationship between gut microbiota and animal behavior. However, other researchers found that administration of antimicrobials to colonized mice reduced the microbial population, and increased exploratory behavior in mice (4). They also found that the same antimicrobials given to germ free mice had no effect on their behavior. Furthermore, they reported that administering the microbial population from colonized mice to germ free mice reduced exploratory behavior in the germ free mice. Such change of exploratory behavior suggests a higher activity of mice when treated with antimicrobials. Though increased exploratory behavior proposes a contrast to a greater fear response, they may actually be complementary depending on the motivation of the exploration (23). However, it is clear that more research needs to be done to determine the impact of postnatal microbial colonization on the development of neural systems that induce stress in animals.

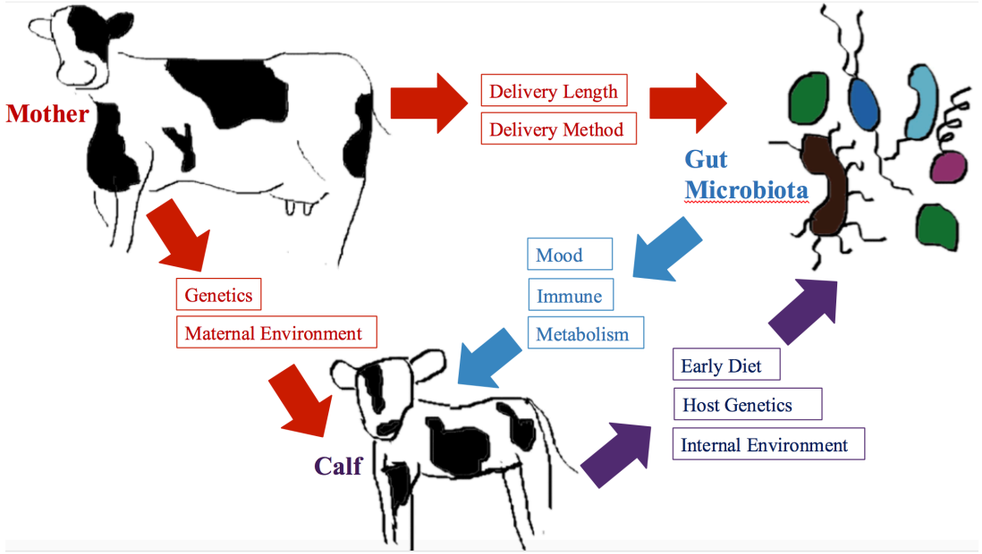

Figure 1. The relationship between the colonization and establishment of the gut microbiota on the early-life calf's immune system, mood and metabolism, shown in blue, and the impact of the calf's early diet, genetics and internal environment on the gut microbiota, shown in purple. Also shown is the maternal impact on the calf by genetics and maternal environment, and the maternal impacts on the microbial colonization of the calf, shown in red.

IMPACT OF THE GUT MICROBIOTA ON DIGESTION AND METABOLIC FUNCTIONS

Recent studies have found that a shift in microbiota during early life can cause obesity, as well as other metabolic diseases like diabetes in later life (19). The study on fecal microbiome of 298 stool samples from 22 children with type 1 diabetes, and 22 normal children of the same age found a significant difference in host-microbial interactions between two groups (6). There was a higher interaction between host and *Enterococcus, Sarcina, Prevotella*, and *Corynebacterium* in the children with type 1 diabetes (7). Relatedly, the authors also found less interaction between *Enterococcus, Sarcina, Prevotella,* and *Corynebacterium* and host in children without type 1 diabetes (7). This correlation of host and bacterial interaction, may suggest that metabolism could be affected by the gut microbiota. To further identify the causal effect of microbiota on host metabolic dysfunction, genetically deficient Toll-like receptor 5 (TLR5) mice were used to induce obesity, and then the obese TLR5 mice microbiota was transplanted to wild type germ free mice (24). Found in the gut mucosa, Toll-Like receptor 5 is a class of receptor involved in innate immune responses, which plays a role in satiety. Without TLR5, the mice exhibited significant hyperphagia, and developed metabolic disease (24). Next, the germ free wild type mice treated with the gut microbiota derived from the TLR5 deficient mice, immediately showing distinct microbial changes, and later becoming obese (24). The change in body composition of germ free mice

from normal to obese after colonized with a specific microbial population, suggests that the gut microbiota may impact body composition through affecting the host metabolic functions.

INITIAL COLONIZATION

Mammals are currently believed to be sterile when *in utero* and microbial colonization can be observed minutes after birth (18). Initial colonization can be defined as the period during, and immediately following birth when microbes first interact with and colonize the sterile infant. Because microbial colonization of the mammalian neonate happens so quickly it is assumed that initial colonization happens from the birth canal or from the first exposure to the environment in neonates delivered by caesarean section (18).

MODE OF DELIVERY: VAGINAL OR CAESAREAN SECTION

Since the mode of initial colonization is thought to happen during the birthing process, it can be assumed that the two different modes of birth, caesarean section or vaginal birth, would result in different infant microbial populations. Indeed, mode of delivery impacts microbial population initially, but the differences minimize with aging (18). Specifically, caesarean section was associated with both lower abundance and diversity of the phyla Actinobacteria and Bacteroidetes, and higher abundance and diversity of Firmicutes from birth to three months of life. It was also found that *Bifidobacterium* and *Bacteroides* genera seems to be more frequent in vaginally delivered infants compared with caesarean section delivered ones (18). However, after six months of age, the microbial differences minimized. Furthermore, it was found that delivery mode was not significantly associated with childhood development of obesity through a longitudinal study from infancy to 7 years of age (2). Though they noted initial differences in microbial population with the different mode of delivery, the differences did not make a lasting impact and by 7 years of age the mode of delivery no longer had an impact on microbial population.

LENGTH OF DELIVERY

Currently, there is no research on the impact of length of delivery on microbial population. The length of delivery can be hypothesized to impact initial microbial colonization because a longer birth would mean the infant spends longer in the birth canal, meaning more exposure to birth canal microbiota. This could be the case, especially in the birthing process of calves. For example, heifers generally have a longer delivery than cows because it is their first time through the birthing process. It is possible that having calves born from heifer or cow may impact the exposure to birth canal microbiota thus impact initial microbial colonization. However, it can be assumed that length of delivery would be similar to type of delivery in that though it may be initially significant, the differences in microbial population may not last into adulthood. However, if there is a difference in length of delivery, it is important to determine if those differences last into adulthood.

FACTORS AFFECTING MICROBIAL ESTABLISHMENT

The establishment of the microbiota is a dynamic process following initial colonization, which depends on factors such as host genetics, diet, maternal environment, early antibiotic exposure, and highly sterile environments (9, 12, 13).

HOST GENETICS

The genetics of the host determine the microenvironment of the gut thus affecting the suitability of the internal environment for microbial colonization. An early study in mice found host genetics had a 12% impact on microbial variation in the gut (25). Additionally, different researchers found a significant link between a mutation in human genes and a corresponding shift in gut microbiota characterized by a depletion of total numbers of bacteria, loss of diversity, and major shifts in bacterial populations within the Bacteroidetes, Firmicutes and Proteobacteria phyla (11). Moreover, another different research group was able to identify a core measurable microbiota of 64 conserved taxonomic groups using quantitative pyrosequencing of the microbiota and individual host genotype had a measurable contribution to microbial variation which can be explained by litter and cohort effects (3). Through further statistical analysis they found suggestive genome-wide linkage with relative abundances of specific microbial taxa, providing clear evidence for the importance of host genetic control in shaping individual microbiome diversity in mammals (3). Although there have been recent linkages between host genetics and microbial population, and microbial population and body composition (2, 3, 17), there has not yet been a genetic connection made between gut microbiota and long-term growth performance such as weight gain.

DIET DURING EARLY LIFE

Differences in microbial population have been found with different diets during early life (9, 12). Breast-fed infants have a gut microbiota dominated by Bifidobacteria, whereas formula fed infants have a more heterogeneous composition and less Bifidobacteria (9). Furthermore, formula with the more closely resembles maternal milk results in a microbial population that is dominated by Bifidobacteria, like breast-fed babies (9, 12, 14, 20). These results suggest that the diet of infants can change the gut microbiota population through the establishment process.

MATERNAL ENVIRONMENT

Like diet, prenatal maternal stress can influence the composition of the infant's gut microbiota. Maternal stress is hypothesized to change the maternal physiological environment, including increased heart rate and introduction of stress hormones (2). Additionally, stress can also change the maternal environment behaviorally by increasing or decreasing the mother's appetite, activity level, and potentially changing the mother's diet. Such change in maternal environment can affect the mother's microbial population, and can potentially influence initial colonization of offspring at birth (8, 26). Maternal stress can cause increase in cytokine concentration and inflammation, possibly influencing the developing fetus. This maternal immune response creates a different environment for the developing fetus potentially influencing, or changing their immune function development. In addition to maternal stress, other maternal states, like maternal fitness, can impact the infant's microbial population. Additionally, researchers have found a high body mass index (BMI) of the pre-pregnancy mother had a significant impact on the development of obesity in the offspring (2). Further, the same study reported that if those children of a high BMI mother were treated with antibiotics early in life, it reduced obesity prevalence in these children (2). Contrastingly, children born to normal

BMI mothers and treated to early antibiotics were seen to have an increased risk of becoming obese later in life (2). Such influence of the maternal body composition on the fetal microbiome can potentially be due to a change in the maternal microbial population influencing initial colonization of the fetus. Further, because when children born from a mother with a high BMI had a decreased chance of developing obesity when they were treated with antibiotics early in life, suggests a potential cause for microbial influence (2). Understanding, and acknowledging the impact of the maternal microbiota on the offspring's microbiota is important when it comes to management and treatment because it is clear that maternal environment has an effect on the infant's microbiota.

KNOWLEDGE GAPS IN STUDYING EARLY LIFE: MICROBIOTA IN CALVES

The microbial colonization in cattle, especially in neonates, has profound impacts on nutrition, health, animal physiology and productivity (6). Recent research outlining the importance of the microbiome in maintaining neonate health makes the microbiota a potential source of treatment for calf diarrhea (1). Nevertheless, information on the calf microbiota is still limited and more research is necessary before the microbiota can be used to limit instances, and possibly even prevent, calf diarrhea. Additionally, food safety (*E. coli* O157 colonizes the young claves) may be another area of possible research into the importance of the microbiome of beef cattle (6). By understanding the process of how neonatal mammals and microbes interact, it is possible to better target future research in order to solve the problem of calf diarrhea, or other related concerns. Two interesting effects of the microbiota on dairy calves worth noting are the effects of antibiotic treatment on calf behaviour, and the maternal control on the calf`s microbiota. Also, another important area of investigation is the long term effects of microbial on weight gain and body composition of cattle.

The effect of the microbiota on behaviour is important to highlight in calves because when calves are treated with antibiotics for calf diarrhea, the antibiotics may reduce their microbial population, which may lead to exaggerated stress response. Therefore, it is important to investigate if these results can be replicated in calves, and potentially investigate a probiotic for calves to reduce their stress response. Especially a link between stress and immune efficiency has also been noted (5). The implications of antimicrobials and probiotics on calf behavior may be important for calf management. Based on these findings, producers may have the options to give calves the probiotics after antibiotic treatment of calf diarrhea.

Another important aspect is the effect of the mother on the calf microbiota colonization, including maternal genetics and maternal environment. The relationship of host genetics to microbial population is important for calves because it suggests that potentially genetic selection, together with a probiotic, could be the solution to treat calf diarrhea. Also, maternal environment is important to note when investigating potential treatments to calf diarrhea because potentially a change in management of pregnant animals may reduce instances of calf diarrhea. However, more research needs to be done to see the influence of the mother's environment, and body composition, on the microbial population of the calf. In addition, it is a common practice to remove dairy calves from their mothers at a very young age, often increasing their risk of microbial infection because of this high stress period. There are many gaps in the knowledge of the calf microbiota that need to be addressed through research before microbial manipulation can be a potential treatment of calf diarrhea. To date, the understanding of the early life colonization in dairy claves is very limited and it is therefore important to research the role of calf gut microbiome in immune system development.

Since beef cattle are raised in less intensive production systems than dairy, there have been different areas of focus on each of them (15). While microbial investigation of dairy cattle has focused mostly on calf diarrhea, and mastitis, or bacterial colonization of the utter, the microbial focus of beef cattle has been on weight gain and feed efficiency. Though dairy and beef cattle are different in production systems, and physiology, each bovine sector may benefit from collaboration. Therefore, it is important to outline some recent advances in the beef industry. Firstly, rumen variation between beef cattle has been noted between animals housed in the same environment and fed the same diet (6). Thus, the genetic component, or possibly another undiscovered factor, influence the establishment of the gut microbiota. However, more research is needed to explain the observed individualized microbial variation. Further, a significant relationship between weight gain and rumen microbiome was discovered when comparing the most and least efficient animals (17). Regardless, more research needs to be done to investigate long term impacts of microbial manipulation on gut health to prevent calf diarrhea, and growth for better weight gain.

CONCLUSION

Due to the financial impacts of calf diarrhea and the increased occurrence of antibiotic resistance, it is important to find way to treat calf diarrhea that does not include the use of preventative antimicrobials (14). It is common for calves in the dairy industry to be given preventative antimicrobial during early life. Recent research has identified the gut microbiota as a potential tool in reducing the incidence of calf diarrhea. However, research on the calf microbiome is limited. Consequently, this review summarized the current understanding of neonatal mice and human microbiota to outline the importance of the microbiota, as well as microbial colonization and establishment. In addition, the knowledge gaps in calves have been identified. However, it was determined that future research is needed to investigate the relationship between early life microbiota and calf immune system, behavior, and metabolism (Figure 1). Additionally, further investigation is needed to identify the response of the calf microbiome to a change in host genetics, birth length, diet, and maternal environment. Though the understanding of mammalian microbiota has had many recent advances, specific information on the calf microbiome is needed before microbial manipulation can be a potential treatment to calf diarrhea. More research is needed in order to understand how the calf gut microbiota may respond to manipulation.

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ISOLATION OF HALOPHILIC BACTERIA FROM INLAND PETROLEUM-PRODUCING WELLS

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ABSTRACT

The goals of this study were to isolate microorganisms from oil well–produced water, identify the microorganisms, and test the microorganisms' salt tolerance. Saltwater collected from two well locations producing from different zones in Jones County, Texas, was spread onto Mannitol Salt Agar (MSA). Isolates showed a 16S rDNA gene sequence identity of 99% with *Idiomarina baltica* and *Marinobacter persicus*. Salt tolerance assays indicated an optimal growth concentration of 10–12.5% NaCl for the *Idiomarina* isolate and a decrease in growth beyond 5% NaCl for the *Marinobacter* isolate. In conclusion, organisms that are phylogenetically similar to marine microorganisms are present in oil well environments, and have variable salt tolerances, which may prove useful in microbial–mediated hydrocarbon bioremediation of high salinity environments.

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KEYWORDS

- *Idiomarina*
- *Marinobacter*
- *Halophile*
- Saltwater
- Petroleum

INTRODUCTION

As of 2015, there were an estimated 1.7 million oil and gas wells currently active in the United States (18). Saltwater is produced as a byproduct of drilling as it is released from the geological formation along with oil and gas. The saltwater was originally sequestered as the respective formations were being laid down at the bottom of shallow oceans in past geological eras (11). In this study, saltwater samples were obtained from the Flippen Limestone and the King Sand formations. The Flippen Limestone is located at a depth of 1850 feet at the drilling location, and is localized to Fisher and Jones County, Texas. The limestone deposition process started in the late Pennsylvanian and continued through the Permian geological time periods (16), approximately 300 million years ago. Sequestered saltwater was released from the limestone upon hydraulic fracturing, a process in which highly pressurized water is used to open fissures within the formation (12). The King Sand was the second formation sampled in this study, and is a Pennsylvanian era channel–sand deposition located in Central Northern Texas (5), and thus slightly older and deeper than the Flippen Limestone, measuring at a depth of 2000 feet at the oil well location. Saltwater produced at these sites is pumped to the surface with oil and gas, where the components are separated and eventually utilized or discarded.

A consequence of the prevalence of oil drilling is hydrocarbon contamination of soils and groundwater. Microbial–mediated bioremediation of hydrocarbon contaminated environments is an effective treatment, however the efficacy is limited by the conditions necessary for the microorganisms to optimally biodegrade hydrocarbons (9). Salinity of the environment, either indigenous or elevated by the contamination event, is an inhibitory condition for microorganisms responsible for the bioremediation (25). Studies of optimum salinities for halophilic, hydrocarbon degrading microorganisms could lead to targeted bioremediation efforts.

Halophilic microorganisms are a type of extremophile which require a high salt content in order to survive. The extent of halotolerance is categorized by the percent salt concentration within the environment required for optimum growth. Slight halophiles exhibit optimum growth at 1–5% NaCl, moderate halophiles at 5–20% NaCl, and extreme halophiles from 20–30% NaCl (10). Halotolerant organisms are resistant to the deleterious effects a high salt content environment poses, but do not require a high salt content for survival. An environment high in salt is inhibitory for most microorganisms, as water from inside a cell will diffuse through the semipermeable cell membrane into the environment *via* osmosis and ultimately result in plasmolysis (3). Halotolerant microorganisms utilize haloadaptation to overcome this challenge, excluding salt from the cytoplasm where possible and biosynthesizing or accumulating organic osmotic solutes to remain isotonic relative to the environment (24).

Due to the range of salinities, temperatures, and possible carbon sources, oil fields and their related infrastructure pose a potential treasure trove of microbial diversity. Previously identified microorganisms isolated from oil well associated environments include *Marinobacter aquaeolei*, which was described after being isolated from the head of an offshore oil rig off the coast of Vung Tau, Vietnam, but which also occurs in the water column of the same area (17). From the same oil field, *Desulfovibri vietnamensis* was isolated from oil storage tanks, as well as oil well produced saltwater (8). Members of the genus *Desulfovibri* have been implicated in the corrosion of oil infrastructure *via* reduction of iron (13). The genus *Petrotoga*, named for its outer toga–like sheath, is associated exclusively with oil production, having been found in offshore wells and inland oil reservoirs (23). The diversity of microorganisms in these salt rich, petroleum associated environments led us to look for variable halophilicity in microbe populations by sampling saltwater produced from different geological formations. In this work, we isolated two microorganisms from saltwater produced as a byproduct of oil drilling, belonging to the genera *Idiomarina* and *Marinobacter*, and characterized their respective salt tolerances.

METHODS

SAMPLING

Permission to sample, access to well sites, and instructions on equipment use were provided by the lease holder, JQL ENERGY, LLC. Saltwater, produced as a byproduct of oil drilling, was collected aseptically from two oil well locations in Jones county, Texas. Locations, shown in Figure 1 were chosen due to contrasting geological characteristics, as the first oil well pumps from a solid limestone formation, while the second pumps from a course-grain sand formation. The Flippen Limestone sample was obtained from a fiberglass saltwater storage tank, while the King Sand sample was obtained from a water knockout, a horizontal tank which separates oil from saltwater based on varying density. Four samples, two from each well site, were collected using 118 mL sterile glass jars and stored overnight at 4°C. The King Sand samples were collected *via* release valve located on the bottom of the water knockout tank. The Flippen Limstone samples were collected *via* an access port on top of the saltwater tank, directly from the separated saltwater stored within. Initial sampling took place in November, 2015. The *Idiomarina* isolate has since been re-isolated, indicating that our method can be replicated.

Figure 1. Map of sampled locations in Jones County, Texas. The Muehlstein lease, located at 32°50'06.29"N, 99°43'51.26 W, elevation 1550 feet, is the location of the sampled saltwater storage tank from which the *Idiomarina* isolate was isolated, and is used to store saltwater pumped from a Flippen Limestone oil field. The Swenson Lease, located at 32°50'48.75" W, elevation 1556 feet, is the location of a sampled water knockout tank from which the *Marinobacter* isolate, and which separates saltwater from oil pumped from a nearby King Sand well.

GROWTH CONDITIONS

Samples of 1.0 ml were plated directly onto Mannitol Salt Agar (MSA) (Hardy Diagnostics, Santa Maria, CA), selected for its 7.5% salt content, and incubated overnight at 37°C. MSA is typically utilized as a selective and differential medium for detection of pathogenic Staphylococci, however it is also suitable for halophilic marine organisms (20). An incubation temperature of 37°C was selected by applying a formula designed to calculate the temperature of a geological formation: Formation Temperature = Surface Temperature + (Temperature Gradient * Vertical Well Depth) (19). The average regional high temperature of Jones County, Texas in November, 2015 was 66 °F, roughly 19°C (6), which when plugged into the formula with the depth of the King Sand well (2000 feet), and a temperature gradient of 0.015 °F/ft., yields a formation temperature of ~36°C. Individual colonies were selected haphazardly and subsequently sub-cultured on MSA.

IDENTIFICATION

To identify the microorganisms, the 16S rDNA genes were amplified and sequenced. DNA was extracted using a Zymo DNA extraction kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's instructions. Recombinant Vent DNA polymerase (New England BioLabs Inc., Ipswich, MA) was used in PCR to amplify a portion of the 16S rDNA gene. PCR was executed utilizing the primers 5'-AGAGTTTGATCCTGGCTCAG-3' (F'-27m) and 5'-TACCTTGTTACGACTT-3' (R'-1492) (Positions 11–27 and 1489–1506, respectively, according to the *Escherichia coli* 16S rRNA numbering system of the International Union of Biochemistry) (14). Primers were designed to our specifications and synthesized by Invitrogen. The thermocycling conditions for amplification were as follows: initial denaturation: 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute 30 seconds followed by a final extension at 72°C for 10 minutes. The products were then confirmed using gel electrophoresis of a 0.8% agarose (Invitrogen; Carlsbad, CA) gel containing ethidium bromide under a 70 to 90 voltage for 30 to 45 minutes. The resulting amplicons of 1.5 kb were further purified using DNA Clean & Concentrator (Zymo Research Corporation, Irvine, CA) and 16S rDNA sequencing was outsourced to DNA Analysis Facility on Science Hill at Yale University (New Haven, CT). The identification of phylogenetic neighbors and the calculations of pairwise 16S rDNA gene sequence similarities were achieved using NCBI BLASTn Analysis (1). Sequence alignment and creation of phylogeny were performed using Molecular Evolution Genetics Analysis 7.

ASSAY

Salt tolerance assays (Figure 2.) were carried out in Tryptic Soy Broth (TSB) (Becton, Dickinson and Company, Sparks, MD); the *Idiomarina* isolate was tested in 0.5-25% NaCl while the *Marinobacter* isolate was tested in 0.5-12.5% NaCl. Culture tubes containing 2 ml of TSB with concentrations of NaCl from 0.5-25.0% were inoculated with 30 µl of an overnight culture and incubated at 37°C for 48 hours with aeration by a shaker at 200 rpm. The optical density at 600 nm (OD600) of four replicates at each NaCl concentration were measured using a Hewlett Packard 8453 UV-Visible Spectrophotometer. Cultures were diluted 2-fold with sterile water to obtain accurate OD600 readings as necessary.

STATISTICAL ANALYSIS

The error bars in Figure 2 indicate standard deviation of measured OD600 between four replicates. Statistical analysis of the OD600 for both data sets was calculated using Microsoft Excel's Data Analysis add-in. An alpha of 0.05 was used as the cutoff for statistical significance. The *Idiomarina* isolate data set has a *p*-value of 0.43238 and is therefore beyond the cutoff for statistical significance, while the *Marinobacter* isolate data set has a *p-value* of 0.00017 and is therefore statistically significant.

RESULTS

Two *Gram-negative*, mesophilic, rodshaped isolates were obtained from saltwater samples originating in different geological formations utilized in oil and gas drilling. The isolate from the Flippen Limestone showed sequence similarity to the genus *Idiomarina*, while the isolate from the King Sand showed sequence similarity to the genus *Marinobacter*. Figures 3 and 4 illustrate phylogenetic relationships between the isolates and closely related species, chosen based on similar figures in the novel species reports of the isolates' closest 16S rDNA identities, *Idiomarina baltica* and *Marinobacter persicus*, respectively.

The *Idiomarina* isolate showed a 99.7% 16S rDNA sequence identity with *Idiomarina baltica*, originally isolated from the central

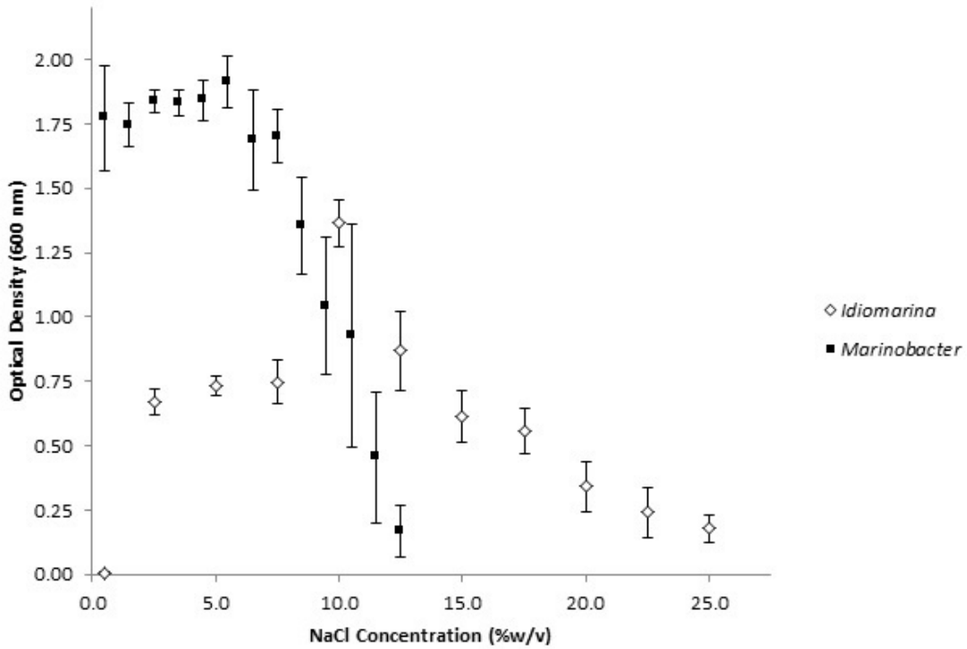

Figure 2. Plot of *Idiomarina* and *Marinobacter* isolate salt tolerance. Tubes containing 2mL TSB with varying concentrations of NaCl were aseptically inoculated and incubated 48 hours at 37 °C with aeration. Optical density at 600 nm was measured using a Hewlett Packard 8453 UV-Visible Spectrophotometer. The y-axis represents the optical density, while the x-axis represents the increasing salt concentration of each tube by weight/volume percent. Error bars indicate standard deviation of the optical densities between four replicates.

Baltic Sea. Morphologically, the isolate produced raised, circular, opaque, mucoid colonies, with white pigmentation that takes on a purple tint as it continues to grow on the MSA. The rod shaped, Gram negative cells were motile by a single polar flagellum. Results from the salt tolerance assay indicate an optimum NaCl concentration between 10-12.5% (Figure 2.), making the isolate a moderate halophile.

The *Marinobacter* isolate showed a 99.1% identity with *Marinobacter persicus*, originally isolated from Lake Aran-Bigdol, a hypersaline lake located in Iran. The isolate grew on MSA overnight incubated at 37°C and produced colonies morphologically characterized as small, convex, circular, mucoid, with a translucent leading edge, and white pigmentation that takes on a purple tint as it continues to grow on MSA. The rod shaped, Gram negative cells were motile by a single polar flagellum. Results from the salt tolerance assay indicated a sharp decrease in growth beyond 5% NaCl (Figure 2.), and the ability to grow in TSB without supplemental salt, making the isolate moderately halotolerant.

DISCUSSION

The identification of the isolates as being moderately halophilic and moderately halotolerant was expected, given the salt-rich environment. However, the close phytological association of the isolates with classically marine bacteria genera was unexpected, as the

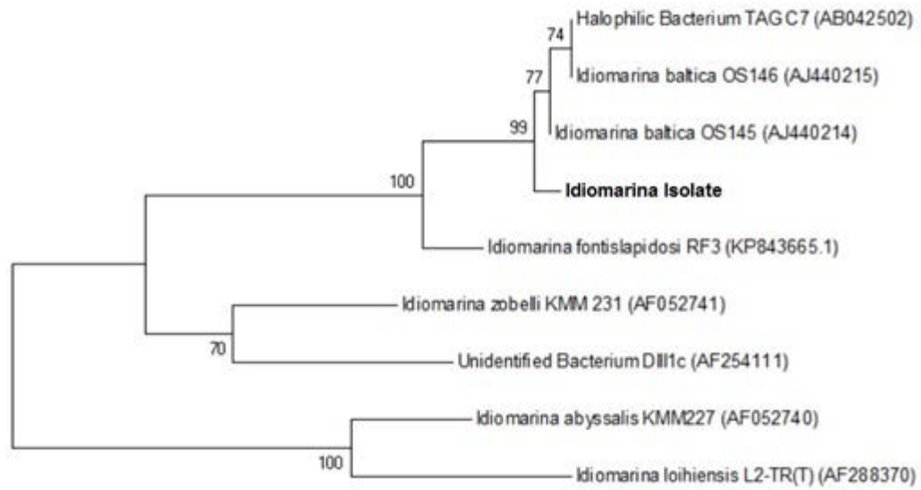

Figure 3. Unrooted phylogenetic tree generated from 16S rDNA sequences of members of the genus *Idiomarina*. Phylogenetic tree illustrates an inferred evolutionary relationships with selected species. Species were selected within the genus *Idiomarina* based on relation to *I. baltica*, our *Idiomarina* isolate's closest 16S rDNA identity match. A neighbor-joining algorithm with 500 bootstrap replications was used to produce the following topology. Alignment and phylogeny preformed using Molecular Evolution Genetics Analysis 7.

oil wells sampled are hundreds of miles inland and draw from reservoirs thousands of feet deep. Given the origin of the saltwater present in these formations, perhaps it should not be unexpected, as a likely explanation is that the seawater was sequestered from ancient seas as the strata of the geological formations were being deposited approximately 300 million years ago. Other less likely possibilities are that the oil wells could have become contaminated from fluid or equipment used to drill the well initially. Drilling rigs and associated equipment move from site to site, increasing chance of cross-contamination. Hydraulic fracturing, which used municipal water from Albany, Texas in both well sites, could have potentially introduced contaminants deep into the formations, and may change the indigenous chloride concentration, as the water is treated with 2% potassium chloride before use.

A contemporary study of two Appalachian Basin shales also found *Idiomarina* and *Marinobacter* members in low abundance during their 328 day metagenomics and metabolic analysis study. Findings from the study include niche differentiation among closely related strains, and interconnected metabolisms of microorganisms persisting in hydraulically fractured shales. A mechanism for aerobic oxidation of alkanes and respiration of sugars *via* nitrate and oxygen electron acceptors is proposed to be utilized by members of the genus *Marinobacter* present in the studied environment (7).

The salt tolerance assay shown in Figure 2. utilized varying optical densities with NaCl concentration as an indicator of microbial growth. As the bacteria have their growth inhibited with changing salt concentration, the optical density decreased as there were less cells present to scatter light from the spectrophotometer. This allowed us to chart the isolates' growth from NaCl concentrations of 0.5% w/v, which is TSB with no supplemental

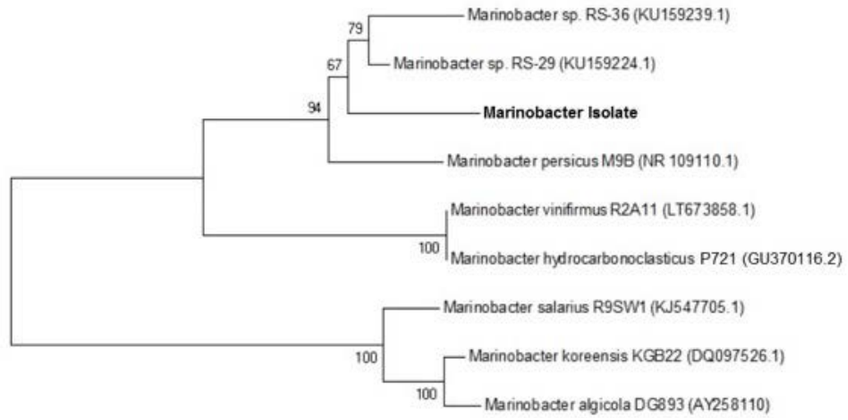

Figure 4. Unrooted phylogenetic tree generated from 16S rDNA sequences of members of the genus *Marinobacter*. Phylogenetic tree illustrates an inferred evolutionary relationships with selected species. Species were selected within the genus *Marinobacter*, based on relation to *M. perscicus*, our *Marinobacter* isolate's closest 16S rDNA identity match.

salt, to a supplemental NaCl concentration of 25% w/v. The results of the experiment indicate decreased growth beyond 5% NaCl for the *Marinobacter* isolate, but the ability to grow with only 0.5% NaCl, indicating that it was moderately halotolerant. The *Idiomarina* isolate could not grow at 0.5% NaCl, and could grow optimally at 10–12.5% NaCl, indicating that it was moderately halophilic.

An optimum salt concentration of 3–6% was initially reported for *Idiomarina baltica*, compared to the *Idiomarina* isolate's 10–12.5% reported here, classifying this strain as moderately halophilic (Figure 2). Morphologically, the two are similar with *Idiomarina baltica* producing colonies characterized as circular, smooth, opaque, and with a slight yellow pigmentation on marine agar. The most similar sequence reported for *Idiomarina baltica* type strain was a 95–96% affiliation with *Idiomarina* zobelli, while our *Idiomarina* isolate showed a closer association with 98% identity to the same species (4). Our *Idiomarina* isolate also showed a 99.2% identity to *Idiomarina Fontislapidosi*, which is reported to grow optimally at 3–5% salt concentration, and is remarkable for being the first member of the genus *Idiomarina* isolated from hypersaline soil rather than water (22). The isolate clusters closely with *Idiomarina Fontislapidosi* and strains OS145 and OS146 of *Idiomarina baltica* (Figure 3.), but has a much higher optimum salt concentration.

An optimum salt concentration of 7.5–10% was previously reported for *Marinobacter persicus*, making it moderately halophilic, while our *Marinobacter* isolate has decreased growth beyond 5% supplemental salt. The two are morphologically different as well, with *Marinobacter persicus* producing colonies characterized as raised, punctiform, contoured, and with a yellow-orange pigmentation when grown on HM agar. At the time of *Marinobacter persicus*'s novel species report, M. hydrocarbonoclasticus was the closest relative with an identity of 97.7%, while our *Marinobacter* isolate shows a 98.2% identity with the same species (2). The isolate also clusters closely with *Marinobacter* sp. RS-29, and *Marinobacter* RS-36 (Figure 4.), isolated from Yuncheng Salt Lake in China (21). Given the sharp decrease in the *Marinobacter* isolate's growth beyond 5% NaCl, it seems likely that the Yuncheng Salt Lake isolates have a higher salt tolerance due to the lake's inherent salt richness (27).

Hydrocarbon degradation abilities notwithstanding, results from the salt tolerance assays (Figure 2.) suggest that the isolates would be suitable for a range of bioremediation efforts that might otherwise be inhibited by a high environmental salt content. The *Idiomarina* isolate could prove useful in scenarios where separation of oil and saltwater does not occur before the contamination event, such as well site contamination due to leaking lines or storage tank failure, which ultimately results in the contaminated area being saltier than a typical oil spill. The *Marinobacter* isolate grows well at a concentration of 3.5% salt, ocean level salinity, and would be well suited to cleaning up hydrocarbon contamination on beaches and marine environments.

In conclusion, halophilic and halotolerant microorganisms phylogenetically associated with microorganisms present in saline and hypersaline bodies of water, such as the ocean and salt lakes, can be found in the saltwater produced as a byproduct of drilling for oil and gas. By sampling different geological formations, we were able to successfully isolate and characterize microorganisms with differing levels of halotolerance. Results indicate the *Idiomarina* isolate was moderately halophilic with an optimal NaCl concentration of 10–12.5%, while the *Marinobacter* isolate, was moderately halotolerant with decreased growth beyond 5% NaCl. Future studies will investigate hydrocarbon degradation by the new isolates, as the related *Idiomarina xiamenensis* is known to act in a hydrocarbon degrading consortium with other marine bacteria (26), and the genus *Marinobacter* has multiple members reported as capable of utilizing hydrocarbons (15).

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ANTIBIOTIC RESISTANT BACTERIA IN AN URBAN FRESHWATER ECOSYSTEM IN CENTRAL TEXAS

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ABSTRACT

Antibiotic resistance is a growing concern for the human population and is becoming prevalent in many environments. For example, increasing occurrences of antibiotic resistance genes (ARGs) in aquatic ecosystems elevates the risk of pathogenic microbes acquiring those resistance genes. There is an urgent need to more closely examine the relationship between antibiotic resistant bacteria (ARB) and antibiotic residues in urban freshwater environments. Thus, our main objective was to investigate the presence of antibiotic resistance in wastewater treatment plant (WWTP) influent and effluent leading into the Brazos River using several commonly used antibiotics: penicillin, ciprofloxacin, erythromycin, trimethoprim, tetracycline, sulfamethoxazole, and gentamicin. An additional aim was to explore possible mechanisms of resistance emergence to these antibiotics using techniques such as replica plating, the Luria–Delbrück Fluctuation Test, the Newcombe Test, and 16S rRNA sequencing. Four samples of influent and treated effluent wastewater were collected from the WWTP to enumerate resistant bacteria in the community and to investigate whether mutations causing resistance in ARB might be induced or spontaneous. We found that penicillin had the highest rate of resistance in all samples and that a similar trend of resistance appeared across all four samples. According to the Luria–Delbrück Fluctuation Test and the Newcombe Test, different antibiotics appear to be associated with different tendencies of resistance emergence, with certain groups of antibiotics producing different results, which raises evolutionary questions about the roles of random mutation and induction. Most ARB detected from the Luria–Delbrück Fluctuation Test belong to the *Klebsiella*, *Enterobacter*, and *Aeromonas* genera. This study provides a baseline understanding of the urban freshwater ecosystem status in central Texas and quantitatively examines the degree of resistance emergence.

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INTRODUCTION

Since the discovery of penicillin in 1928 (13, 14), antibiotics have played a crucial role in the fight against pathogens and infections, as well as increasing livestock growth and health (41). However, recent concerns have become more pressing due to the increasing number of antibiotic resistant pathogens in medical settings across the world – resulting in the loss of *via*ble treatment methods. In an attempt to stifle the alarming rate at which antibiotic resistance has been occurring, researchers have begun to investigate the role of the environment in the spread of antibiotic resistance to both human and animal populations (23). Modern wastewater treatment plants (WWTPs) have been found to serve as an important source of antibiotic resistant bacteria (ARB) and antibiotics (12, 22). The primary goals of WWTPs are to remove solids, reduce numbers of pathogens, and sequester nutrients such as organic carbon, nitrogen, phosphorous, and fatty acids, and are not designed to remove antibiotics and other pharmaceuticals that ultimately pollute receiving bodies of water (15, 24, 44). Some of these remaining antibiotics are minimally biodegradable in freshwater ecosystems (1), which can result in antibiotic residues of variable concentrations with unknown selective consequences for environmental bacteria (17).

The presence of antibiotics can select for bacteria already carrying antibiotic resistant genes (ARGs), allowing for preferential growth and propagation of their genome through horizontal gene transfer (HGT) and asexual reproduction (3, 28). There have been numerous studies within the last several decades which present data on the exacerbation of antibiotic resistance in microbial communities in various sources of freshwater as a result of improperly treated run-offs from large facilities such as hospitals, pharmaceutical production factories, and livestock farms (4, 9, 33, 36, 37). In addition to the problem of antibiotic and pharmaceutical compounds, many treatment measures used in WWTPs may not be effective in removing resistant microbes themselves from sewage influent (18, 25, 29). This allows bacterial contaminants to be flushed into receiving bodies of freshwater (20, 31, 41, 45). These bacterial contaminants increase the likelihood of spreading ARGs by residing in environments with potential for frequent HGT to downstream aquatic microbiota.

This cycle produces an accumulation of ARGs and ARB, allowing urban water sources to serve as both reservoirs and breeding grounds of resistance (2). Urban aquatic ecosystems in Brazil have demonstrated communities capable of tolerating antibiotic concentrations up to 600 times higher than levels in clinical usage (11). ARGs in aquatic microbes may be relatively harmless to humans when found in non–pathogens, but they can be transferred to pathogens or human and animal commensals (7, 12). When resistance occurs, especially in opportunistic pathogens, it can serve as a risk to human health when affected freshwater sources are used for human consumption and recreation. Infections become increasingly dangerous as readily available and widely used treatments may no longer be effective (6) with the accumulation and spread of antibiotic resistance.

Many of the aforementioned studies in freshwater ARB and ARG presence have been conducted in Europe and Asia, however, there have been no such studies for urban aquatic ecosystems in central Texas. This research gap

highlights the lack of information on antibiotic presence and resistance in important Texas watersheds, and as a result, the severity of ARB proliferation needs to be studied to gain a grasp on the current situation. The research objective was to investigate the current status of ARB in a central Texas urban aquatic ecosystem, and the possibility of antibiotics in WWTP influent and effluent genetically influencing and selecting for ARB in an urban freshwater environment. We hypothesized that antibiotic resistance would be present in the samples collected from the WWTP and that mutation tests will show a prominent trend of spontaneous mutation in resistance.

METHODS

SAMPLING

Water samples were collected from the Waco Metropolitan Area Regional Sewage System (WMARSS). WMARSS is a joint wastewater treatment plant that serves eight urban cities with an average flow of 37.8 million gallons per day. The effluent from the treatment plant leads into the Brazos River. In addition to the WMARSS, other local watersheds entering the Brazos River near Waco include several cattle pastures, other smaller treatment subsets of WMARRS, and an artificial wetland ecosystem constructed by the city of Waco north of the Brazos River.

Four 500 mL samples of influent and effluent were collected in Nalgene™ Lab Quality Amber HDPE Wide Mouth bottles. The bottles were machine washed, rinsed 3X with distilled water, treated with a 10% sodium hypochlorite solution, then rinsed thoroughly with molecular-grade purified and filtered water. This was considered adequate to remove not only potential dust-borne or water-borne contaminating bacteria, but extracellular DNA is eliminated by the sodium hypochlorite treatment. The samples were placed on ice immediately after collection and transported to the laboratory, kept refrigerated (-4 °C), and were processed within 48 hours. Sampling was carried out over four weeks, taken once a week in the afternoon.

ISOLATION OF ANTIBIOTIC RESISTANCE BACTERIA

Two types of media were used: Trypticase Soy Agar (TSA) and Eosin Methylene Blue (EMB). TSA is considered a nonselective, general-purpose medium, thus it was used to isolate a wide range of culturable bacteria. EMB was used to enumerate coliform growth. 100 µL of these dilutions were spread plated onto one TSA and one EMB plate, respectively. Since effluent contains far fewer bacteria, 250 mL of each effluent sample was filtered on Pall GN-6 Metricel® MCE Membrane Disc Filters to concentrate the bacteria. The filter was then vortexed for 5 minutes at full speed in conical polypropylene tubes with 10 mL of the respective effluent sample to release the bacteria into the solution. 10 microliters of this solution plated onto one TSA and one EMB media plate. These four plates comprised the master plates for each sampling date.

All EMB plates were incubated for 24 hours at 37°C and all TSA plates for 24 hours at room temperature. TSA plates were incubated at room temperature to more closely approximate the average temperatures encountered by bacteria in effluent and in the Brazos River. Wastewater effluent and river water temperatures are quite variable, and depend on many factors, including time of year, depth of water, flow rate, shade available, etc. For approximately half of the year, surface temperatures of the Brazos River are below 25 oC (data available at https://waterdata.usgs.gov/nwis/uv?08117300). In order to facilitate the growth of a greater diversity of environmental

bacteria, the TSA samples were incubated at room temperature (generally between 20 and 25°C). Although identification of resistant potential human pathogenic coliforms in the WWTP and effluent were a primary concern, it was useful to have a non-selective culture for comparison to the coliform-selective EMB. In addition, some types of resistance that may be harbored by non-pathogenic environmental bacteria have potential for HGT to human pathogens (23, 44).

Seven classes of antibiotics were chosen based on common use in medicine and agriculture, and were used to create antibiotic infused TSA and EMB media culturing plates. Concentration values for each antibiotic (Table 1) were referenced from minimum bactericidal concentrations (MBCs) determined by an article that has established MBCs for a common gut microbe (19). For each type of sample master plate – influent-TSA, influent-EMB, effluent-TSA, effluent-EMB – seven antibiotics were placed on sterile TSA and EMB media culturing plates and left for several hours to sit and absorb into the agar. Using velvet, the master plates were replica plated onto the antibiotic infused plates. A total of 28 antibiotic plates were incubated for 24 hours, at 37°C for EMB plates and room temperature for TSA plates. Bacterial colony growth on each antibiotic plate were deemed resistant to the specific antibiotic and recorded.

THE LURIA-DELBRÜCK FLUCTUATION TEST

During the isolation process, each set of antibiotic infused plates were compared to their respective master plates and a colony that was observed to be susceptible to all seven antibiotics was chosen from each master plate. A first round of pure culture was inoculated on a single plate of respective media using this colony, incubated for 24 hours as before on EMB and TSA plates. Using a random colony from the first round, a second round of pure culture was inoculated on a single plate of respective media and incubated again with the same conditions. This process was repeated, using the previous round to inoculate the next round, until the fifth round. The fifth round was used to inoculate seven antibiotic infused media plates and then incubated. This last procedure was replicated five times to produce 35 plates, and the number of antibiotic resistant colonies were observed and recorded. The Luria–Delbrück experiment intends to test two possibilities: induced or spontaneous mutation (26, 39). The repeated culturing between each generation provides time for mutation to occur during cell division. If the mechanism for antibiotic resistance in bacteria is induction by antibiotics, the number of colonies between the five sets of antibiotic infused plates from the final step should not vary greatly. However, if antibiotic resistance in bacteria is due to spontaneous mutation, then a mutation can occur at any point in the culturing process – either in earlier generations or later generations. This should produce a large amount of variance in colony number between each set of antibiotic infused plates. Levene's test of equality of variances was used to test the significance of results from the Luria-Delbrück test.

THE NEWCOMBE TEST

The fifth round from each respective sample and plate was used to additionally inoculate seven respective antibiotic infused plates. The plates were incubated at their respective temperatures for 24 hours and then re-spread before being incubated for another additional 24 hours. If the colonies and bacterial cells had spontaneously mutated prior to exposure to the antibiotics, then the re-spread plate should have a higher number of bacteria present due to the moved bacterial cells forming new colonies of their own (32). Analysis of variance (ANOVA) was used to test the significance of

results from the Newcombe test.

16S rRNA GENE SEQUENCING

Using the Qiagen DNeasy® Blood and Tissue Kit, DNA was extracted from resistant isolates of the final sets of antibiotic infused plates from the Luria–Delbrück's experiment cultured in BD Difco™ Nutrient Broth. The extracted DNA was run on 1% agarose gel at 85V at 115mA for 45 minutes, stained with Gel–Red (Biotium, Inc.) in order to ensure bacterial DNA was intact. Using the isolated DNA, a 25 µL PCR reaction mixture consisting of 200 µM primers (universal primers set 27F–1492R), 1 µL of template DNA (~1 ug), 9.5 µL of DNA safe water, and 12.5 µL of Amresco® Hot Start PCR–toGel TAQ PCR Master Mix 2X was made for each sample.

The PCR was run on the Applied Biosystems® Veriti ® 96–Well Thermal Cycler, beginning at 96°C for five minutes, then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for one minute, and extension at 72°C for two minutes. The PCR was finished with a seven–minute final extension at 72°C. Products were run on a 1% agarose gel at 85V at 115mA for 45 minutes to ensure presence, with quantity confirmed using NanoDrop 2000 (Thermo Scientific). PCR products were sent for sequencing to Macrogen USA (Rockville, MD), and the sequence results were analyzed through the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) and the Ribosomal Database Project's (RDP) SeqMatch tool.

RESULTS

ISOLATION OF ANTIBIOTIC RESISTANT BACTERIA

In the replicate–plate antibiotic resistance, total culturable count of ARB drastically decreased post–treatment in effluent in comparison to influent (Figure 1.a). However, while overall culturable count of the samples had decreased in the effluent by 10^7 CFU/mL, percentages of resistant bacterial colonies in comparison to total bacterial colonies on the master plate were comparable (Figure 1.b). The penicillin–infused media agar plates had the highest percentage of surviving bacteria grown across all four types of samples when compared to the master plates. Resistance to ciprofloxacin produced the second highest percentages among the samples, with the exception of influent–EMB. Erythromycin–infused media agar plates generally had the third highest percentage of surviving bacteria followed loosely by trimethoprim, tetracycline, and sulfamethoxazole. Gentamicin–infused media agar plates had the lowest percentages of resistance.

THE LURIA–DELBRÜCK FLUCTUATION TEST

Bacterial colony forming units (CFUs) were observed and recorded across all five sets of plates within each sampling group. CFUs of respective antibiotic–infused plates from each set were averaged and the deviation of each plate from the mean was calculated. To display the variability in resistant colonies for each antibiotic, the range of bacterial colony count deviation from the mean was used and the significance was tested using Levene's test (Figure 2). The range in bacterial CFU among the antibiotic–infused plates

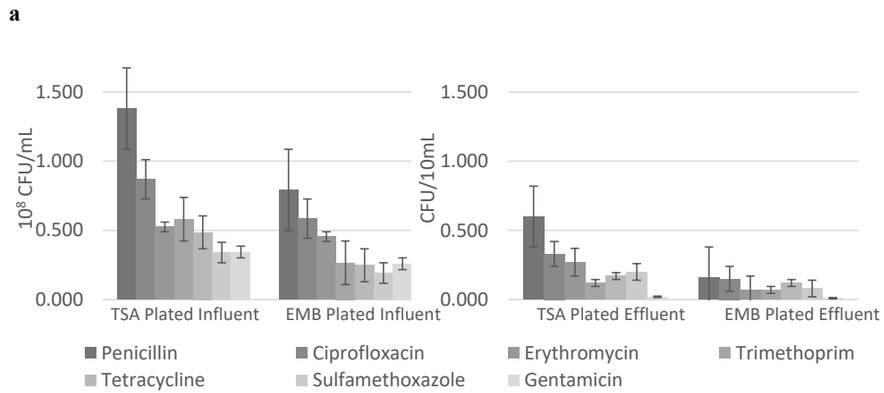

Figure 1. Results for culturable antibiotic resistant bacteria (ARB) for (a) total ARB count (CFU per unit volume), and (b) percentage of ARB in comparison to the master plates from the replicate plate antibiotic resistance assay. Error bars indicate standard error of the means.

did not have a clear trend, with ranges for each antibiotic sometimes varying widely. However, trimethoprim, tetracycline and sulfamethoxazole produced consistently low range values in comparison to other antibiotics. Levene's test showed significant differences in variances between the lower variance (trimethroprim, tetracycline and sulfamethoxazole) and the higher variance group (rest of antibiotics); in which only influent samples on TSA media had marginal significance ($p = 0.070$) while other set of samples showed much stronger significance ($p < 0.001$).

THE NEWCOMBE TEST

In order to compare the number of CFUs on plates before and after the re–spreading process, the difference in the number of colonies formed before spreading and after spreading was calculated (Figure 3). Overall tetracycline, trimethoprim and sulfamethoxazole are among the lowest in CFU difference, but there was no statistical significance between this group from the rest of antibiotics. An ANOVA was not able to reject the null hypothesis for the global

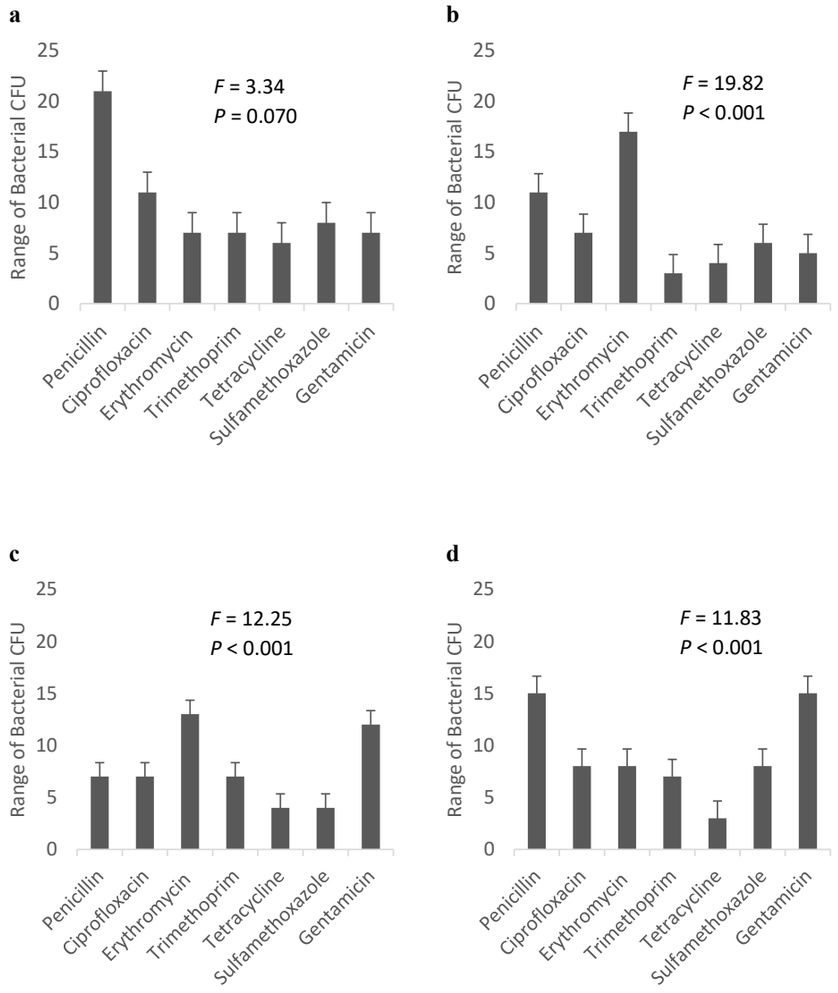

Figure 2. Range of individual plate counts across respective antibiotic-infused plates from the Luria–Delbrück Fluctuation Test. Accompanied statistical results are by Levene's test of equality of variances between lower and higher variance group. (a) TSA Plated Influent (b) EMB Plated Influent (c) TSA Plated Effluent (d) EMB Plated Effluent. Error bars indicate standard error of the mean.

test of significance as well as the pairwise comparisons (Tukey's method) at $p = 0.05$.

16S rRNA GENE SEQUENCING

DNA extracted from bacteria was visualized as bands on the Gel-Red stained agarose gel. Using NCBI's BLAST and RDP's SeqMatch tool, 16S rRNA gene sequencing results from the final round of the Luria–Delbrück Fluctuation Test were analyzed. Gentamicin infused plates did not produce the growth of any resistant isolates. All identified bacteria fell under the phylum α –Proteobacteria, and included the genera *Klebsiella*, *Enterobacter*, and *Aeromonas* (Figure 4). Several isolates were identified at species level (Table 2).

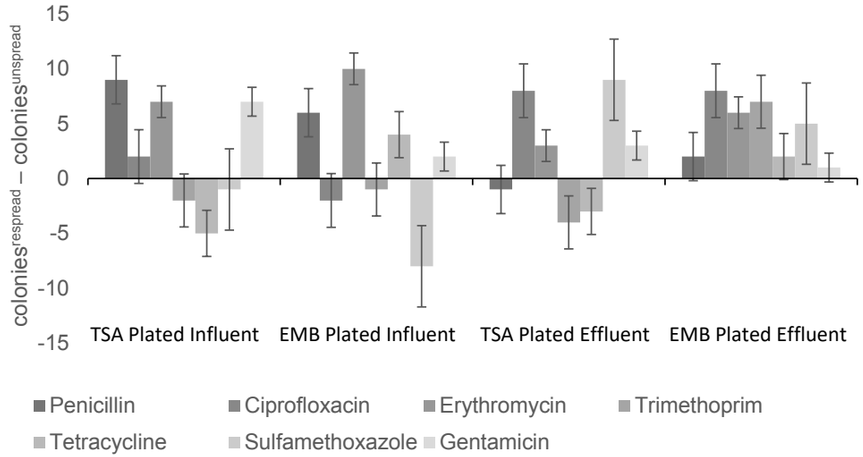

Figure 3. Difference between colony counts for re-spread vs. un-spread plates from the Newcombe Test. A negative value indicates a possibility for induced mutation and a positive value indicates a possibility for spontaneous mutation. Error bars indicate standard error of the means.

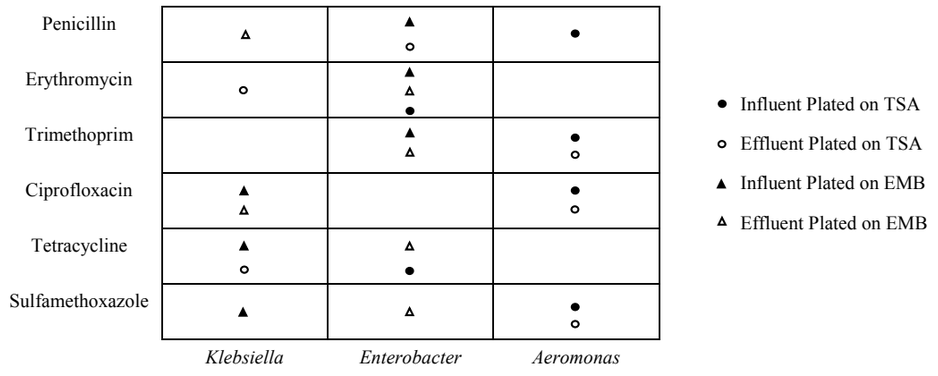

Figure 4. Distribution of identified antibiotic resistant bacteria (ARB) by 16S rRNA gene sequencing per samples and media from the Luria–Delbrück Fluctuation Test.

DISCUSSION

We carried out these experiments to investigate the current situation of antibiotic resistance in a central Texas urban freshwater ecosystem, particularly in an area of the Brazos River where the WMARRS operation releases the effluent. Minimal bactericidal concentrations (MBCs) of antibiotics in media were used to produce a selective environment in which resistant bacteria would be able to survive and grow, allowing for the observation of active ARB. Results demonstrated that antibiotic resistance was clearly exhibited in influent sewage leading into the treatment plant as well as treated effluent water leading into the Brazos River, in both general media and coliform-selective media (Figure 1). The most notable resistance was to members of common antibiotic classes, including a penicillin, a fluoroquinolone, and a macrolide.

It is notable that the third most common type of antibiotic resistant bacteria in TSA-plated effluent are resistant to an antibiotic that has been (repeatedly) detected in WMARRS effluent – erythromycin (Bryan Brooks, personal communication). Fluoroquinolone and macrolide were not detected in the effluent, although bacteria resistant to them were among the most abundant. It is possible that there may be temporal variations in antibiotic concentration, and the particular sampling might have missed the overall trend which may be responsible for the high resistant bacteria abundance. Further studies including that possibility are being planned. Treatment provided by the WWTP was still effective in reducing overall total culturable ARB between influent and released effluent.

Despite being an effective in reducing solid wastes, nutrients, and pathogenic microbes, the WMARRS, as with most WWTPs (18, 25, 29), did not completely eliminate ARB in the effluent samples. In fact, a higher percentage of ARB were found in the effluent compared to the influent in some samples, which has also been reported by previous studies (18, 25, 29), possibly due to the increased opportunity of horizontal gene transfer within the WWTP. This presents an alarming possibility, as accumulation of resistant bacteria in this freshwater ecosystem can potentially create a significant reservoir for the spread and persistence of antibiotic resistance in the environment. As most WWTPs are not designed to remove antibiotics, pharmaceuticals, and other personal care products, the ineffectiveness of reducing antibiotics have been noted in other areas in the world (5, 8, 10, 30).

The detection of antibiotic residues in the WWTP indicates the possibility that at some point prior to reaching the plant, antibiotic concentrations may be high enough for an increased mutation rate, in addition to selective pressure, increasing the prevalence of antibiotic resistance in the environment. Mutation tests, the Luria–Delbrück Fluctuation Test and the Newcombe Test, were used to test this possibility and to serve as a premise for any future experiments to come. In the Luria–Delbrück Fluctuation Test, a lower range in deviation from mean colony count would indicate an induction mechanism due to antibiotic selective pressures, whereas a larger range in deviation would indicate spontaneous mutation occurring through replication and cell division. Although there was no significant trend for the antibiotic residues tested, some antibiotics such as trimethoprim, tetracycline and sulfamethoxazole may be more predisposed to being induced as they produced consistently similar colony counts (Figure 2). Additionally, the fact that antibiotic resistance emerged from antibiotics at MBCs with five rounds of incubation indicates realistic possibilities in the aquatic environment in which conditions are not far from what was used in the Luria–Delbrück Fluctuation Test. Results from the Newcombe Test were not conclusive (Figure 3). After taking the difference between number of colonies on the un-spread plates and the re-spread plates, a positive number indicated that the re-spread plate had a greater number of colony forming units. A higher difference would be indicative of spontaneous mutation, whereas a lower difference would be indicative of induced mutation. Again, the lowest numbers in CFU differences were found with trimethoprim, tetracycline and sulfamethoxazole. There was no clear trend between samples and media, which may have been due to the inadequate number of replicates, which points to the need for follow-up experimentation to determine whether the presence of antibiotics and other factors could be altering the mutation rate leading to these resistance genotypes.

In the final part of the study, 16S rRNA gene sequencing was used to provide a potential list of ARB that have emerged quickly under strong antibiotic concentration pressures. The presence of some

α-Proteobacteria was not surprising, as it is one of the main bacterial phyla present in the human gut microbiome (21, 42). However, both the TSA and EMB media may have been more selective than was originally anticipated, resulting in little diversity in the isolated cultures. Among the identified species, many were opportunistic and commensal pathogenic microbes, and some 16S sequences matched multidrug resistant strains. Influent plated on penicillin- and ciprofloxacin-infused TSA media, and effluent plated on sulfamethoxazole-infused EMB media, grew bacteria that were identified as *Aeromonas* jandaei strain ASH05 (GenBank accession number KU725738), a multi-drug resistant pathogenic strain isolated post-flood in Chennai, India (unpublished). A species of *Aeromonas* identified in influent cultured on trimethoprim- and ciprofloxacin-infused TSA plates, and in effluent cultured on sulfamethoxazole-infused TSA plates (GenBank accession number EU260204), has been referenced in a study examining the antimicrobial resistance in *Gram-negative* bacteria in a lake under heavy anthropogenic influence (34). In effluent cultured on penicillin-infused TSA, an environmental *Enterobacter* species (GenBank accession number EU420931) has been cited in a study exploring the incidence of extended spectrum beta-lactamases (ESBL), and plasmid-mediated AmpC *beta-lactamase* genes and integrons in a eutrophic bay (unpublished). Sequence matches for *Klebsiella pneumoniae* involving multi-drug resistance and nosocomial infections (GenBank accession numbers CP019772, CP017985, CP015392) were found from influent bacteria isolated on tetracycline and sulfamethoxazole –infused EMB media (35). Effluent cultured on ciprofloxacin-infused EMB media produced matches with identified strains of *Klebsiella pneumoniae* that exhibit antibiotic resistance in the environment (GenBank accession numbers KJ806466 and KP297443).

A wide variety of putatively identified strains isolated in this experiment had resistance profiles which matched antibiotics detected at WMARRS; and with strains known for multiple antibiotic resistance, common presence in WWTPs, and pathogenic potential (including nosocomial infections). All of the identified bacteria are in the clinically relevant genera Klebsiella, *Enterobacter*, and *Aeromonas*, including sequence matches to species known for potentially lethal virulence factors (16, 37). This supports the idea that the natural environment and WWTPs can be a significant reservoir for the exchange and maintenance of antibiotic resistance, through horizontal gene transfer and/or selective pressures (26, 42). Despite antibiotic concentrations used in this study being minimum bactericidal concentrations for common gut microbes, numerous microbes were still cultured in effluent samples leading into a freshwater ecosystem. If freshwater sources containing multi-drug resistant pathogens are intended for anthropogenic use, it can serve as an alarming issue. Recently, a study has examined drinking water in six states in the United States and identified the presence of CTXM (an extended-spectrum β-lactamase) and OXA-48 (a carbapenemase) genes (40).

Using culturing techniques, antibiotic resistant bacteria have been characterized at a WWTP in central Texas and the freshwater ecosystem receiving its effluent. There are substantial percentages of bacteria discovered to be resistant to most antibiotics, penicillin being the most abundant and gentamicin being the last abundant. The Luria–Delbrück Fluctuation and Newcombe Tests indicate certain antibiotics having particular mechanisms of mutation and evolution, or that resistance may arise through a mixture of induction and random mutation. The identified resistant isolates that rapidly emerged from the experiment have presented findings that include known pathogens with multi-drug

resistance, which imposes this WWTP and urban aquatic ecosystem in central Texas as a potential risk. Future work in this area includes more detailed examinations of mutation emergence through more extensive mutation tests, general gene sequencing of samples taken from WMARRS, looking at other freshwater ecosystems affected by anthropogenic activity in central Texas, and examining other commonly used antibiotics as selective pressures in resistance reservoirs.

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This research is in compliance with institutional policies relating to infectious agents.

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PREDICTIONS OF FUTURE GEOGRAPHICAL DISTRIBUTION OF TWO VECTORS OF AMERICAN TRYPANOSOMIASIS: IMPLICATIONS FOR ENDEMIC CHAGAS DISEASE IN TEXAS, USA

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ABSTRACT

It is known that climate has a direct effect on vector-borne and zoonotic diseases, and in the face of climate change, understanding this link has become more urgent. Many such vector-borne diseases primarily afflict impoverished populations and have therefore been previously understudied. One major focus of our research is to understand the influence that climate has on the distribution of disease causing microorganisms and their vectors, especially those in relation to American trypanosomiasis (Chagas disease). Chagas disease is caused by the hemoflagellate protozoan parasite, *Trypanosoma cruzi*. For this study, we hypothesized that the increasing prevalence Chagas in the state of Texas is due to expanding distributions of vectors. To test this hypothesis, historical data on vector distribution and climate was used to determine the probable locations of prevalent vectors in Texas. Predictions for the future distributions were made using environmental niche models for bioclimatic variables with a maximum entropy algorithm. Of the two Triatominae species studied, the range and concentration of both decreased under a global warming scenario, a finding that is consistent with the current research of risk of Chagas disease in Venezuela. In future, this same procedure will be used on more Chagas vectors to better understand if there is a northward shift for vectors, or if Texas is becoming more inhospitable to all vectors of Chagas.

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KEYWORDS

- *T. cruzi*
- Chagas disease
- Triatomine
- Climate Change
- Vector

INTRODUCTION

It has long been understood that the Earth's climate has wide ranging impacts on human health (9). One such impact that has been previously understudied is the effect climate has on microbial diseases. Changes in temperature and rainfall, as we are currently seeing in anthropogenic climate change, may affect the distribution and abundance of disease vectors (9).

These vectors may carry microbial agents of disease, such as viruses (causative agents of diseases such as Chikungunya, Dengue, and West Nile Virus), bacteria (causative agents of diseases such as Lyme Disease), and protozoans (causative agents of diseases such as Leishmaniasis) (7). The focus of this study is the vector-borne disease American trypanosomiasis, or Chagas disease.

Chagas disease is a tropical infectious disease that is estimated to have affected as many as 18 million people worldwide, with more conservative estimates placing the number in the 8-10 million range (18, 12, 13, 7, 20). The majority of human infections occur in Mexico and Central and South America (1, 18). The variable estimates of disease prevalence are caused by underreporting of infection, often due to the fact that many people do not know they are infected and do not seek treatment (12, 5). This is, in part, due to the dual phased nature of the disease (1). The acute and the chronic phase are often separated by several decades (18). The first phase (the acute phase) may last anywhere from a few weeks to several months after initial infection (12, 18). The acute phase is asymptomatic or characterized by mild flu-like symptoms not unique to Chagas, including fever, fatigue, body aches, headache, rash, loss of appetite, diarrhea, and vomiting (12, 18). The chronic phase, which develops in 30% of all people infected, may exhibit cardiac or gastrointestinal (GI) complications and can be life threatening (12, 18). Common cardiac signs include cardiomyopathy, heart failure, altered heart rhythm, and cardiac arrest/sudden death (12, 18). Less commonly, chronic GI symptoms include enlarged esophagus (megaesophagus) and enlarged colon (megacolon), both of which can lead to difficulties with eating or passing food (18). While there are cures for Chagas, they are most effective in the acute phase (18). Symptom management and treatments in the chronic phase may inhibit the progression of cardiomyopathy and lower the fatality rate, but the evidence for their efficacy is weak (18).

Chagas disease is caused by the hemoflagellate protozoan parasite *Trypanosoma cruzi* (12, 20). Infection may occur *via* organ transplants and blood transfusions from infected donors, and the consumption of uncooked food contaminated with feces from infected bugs and congenital transmission (12, 22). However, the most common route is vector transmission (12). *T. cruzi* lives in the gut of a variety of insects in the trypomastigote form, and is shed in the feces of the insect vector (4, 2, 4, 23). The parasite is then able to enter the bloodstream of a host *via* the wound left by the insect vector's bite. Once in the host cells, *T. cruzi* transforms from a trypomastigote, a mature flagellate form of the trypanosome, to an amastigote, when no external cilia or flagella are present, and multiplies within the tissue (4). Progeny are released as trypomastigotes into the blood, to spread the infection to other tissues within the current host (4). While trypomastigotes are in the blood the host can serve as a reservoir, and any uninfected insect vectors that take a blood meal from this host may become infected, thus perpetuating infection to other organisms (4). The insect vectors, all from the Triatominae family, are commonly called kissing bugs, due to their tendency to bite around the lips and eyes (1, 22, 7). In the endemic areas of the disease range, approximately 50% of permissive vectors (vectors capable of transmitting disease to humans) carry detectable burdens of *T. cruzi* (5, 20). The most common species that transmit Chagas are *Triatoma sanguisuga*, *Triatoma gerstaeckeri*, *Triatoma protracta*, and *Rhodinus prolixus* (5, 7, 20). *T. gerstaeckeri* and *T. sanguisuga* were chosen to be the focus of the study as they are among the most common Chagas vectors in Texas, and are commonly linked with human

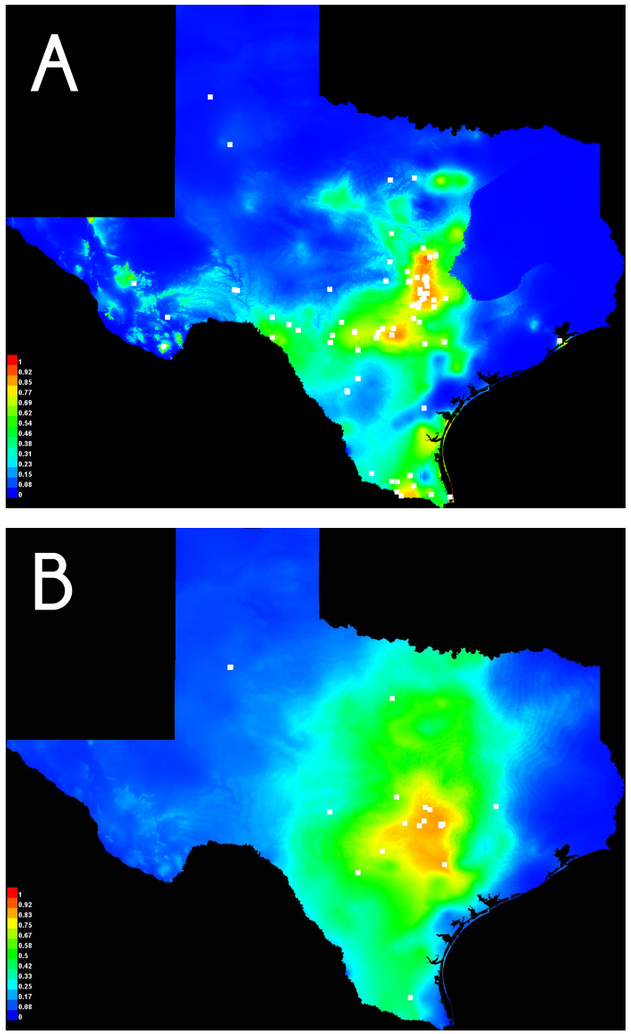

Figure 1. Maxent model for distribution under current conditions (average of 1960 to present). White dots are training locations, warmer colors indicate higher risk of vectors being present for A) *Triatoma gerstaeckeri* and B) *Triatoma sanguisuga*

and canine *T. cruzi* infection. *T. gerstaeckeri* has the highest *T. cruzi* infection rate in Texas, with 58% of all *T. gerstaeckei* individuals testing positive, making it a clinically relevant vector (5, 7).

Historically, endemic Chagas disease has been limited to rural areas of Mexico, Central America, and South America (1, 18). This range has been maintained due to the suitable environment for the reservoirs, Triatominae vectors, and the *T. cruzi* parasite (12, 20). Relevant reservoirs in Texas include dogs, feral swine, woodrats, and armadillos (12). Ideal climate parameters for the transmission of the parasite are tropical conditions, with mild winters, high humidity, and warm nights (1). With changing climates and global warming, there has been a recent shift in the distribution of vectors, including those transmitting diseases such as malaria, lyme disease, leishmaniasis, as well as Chagas disease

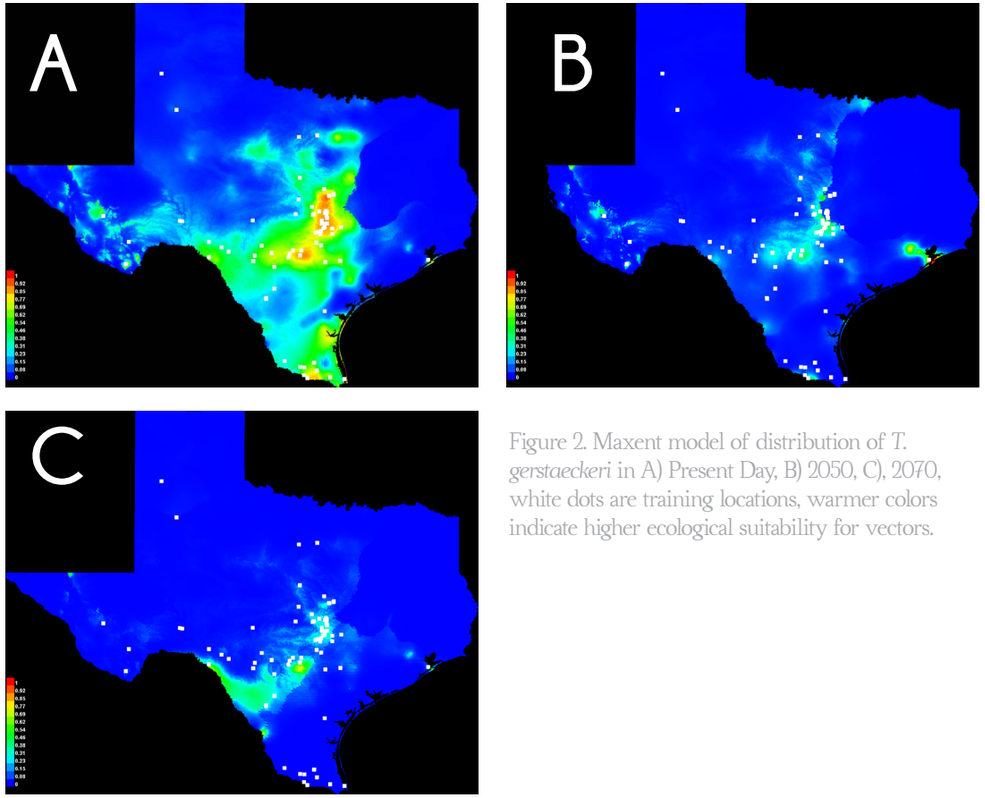

Figure 2. Maxent model of distribution of *T. gerstaeckeri* in A) Present Day, B) 2050, C), 2070, white dots are training locations, warmer colors indicate higher ecological suitability for vectors.

(1, 6, 20).

The southern United States are already suitable habitats for the kissing bug vectors, as seen in recent autochthonous cases (cases acquired within the United States) of Chagas disease (12). As the disease is underdiagnosed and unreported, estimates of the true prevalence of Chagas in the southern United States vary wildly. In Texas alone, estimates of infection range from 4 to 267,000 total locally acquired cases (1,15). The large range of suspected cases exists because Chagas is often underdiagnosed and untreated. The two phase course of the disease, nonspecific symptoms, and possibility of totally asymptomatic infection makes diagnosis difficult (12). Surveillance of donated blood supply in Texas has highlighted that there are a number of Chagas disease cases that were never treated. In Texas, 0.01% of donated blood tested positive for *T. cruzi*. Texas leads the United States in autochthonous cases, reported mainly in the southern regions of the state (12). The first autochthonous case of Chagas disease in the United States was in southern Texas in 1955, and since then the number of locally acquired cases have continued to grow. However, most studies use seroprevalence, which does not differentiate between locally acquired cases and those that were contracted in traditionally endemic countries. No recent publications specifically assessed locally acquired infection in a larger setting than individual case reports. While human data is far from complete, the use of sentinel species, including dogs, also shows an increasing number of cases over time since the mid 20th century. This

trend is expected to continue, with more and more cases occurring in the U.S. with the highest risk in Texas (14). The main goal of this project is to assess the impact climate change is projected to have on bioclimatic variables in the years 2050 and 2070, and if this will change the ecological suitability of Texas for *T. gerstaeckeri* and *T. sanguisuga*. To explain the increase in locally acquired Chagas disease cases, we predicted that the ranges and concentrations of our species of interest would increase. However, our results do not agree with this prediction, indicating that the future Chagas cases in Texas are more likely to be caused by species that are coming to Texas from Latin and South America in response to a changing climate worldwide.

METHODS

DATA

Data of the occurrence and distribution of specific triatomine vectors was assembled from previous collections dating from present day to 1960. Records of previously collected, identified, and *T. cruzi* tested specimens were obtained from museum collections (Texas A&M University, College Station; and University of Texas Brackenridge Field Laboratory, Austin), and published peer-review journal articles (2, 5, 6, 7, 10, 11, 17, 20, 23, 14). Only collections post-1960 were used because the WorldClim (Version 1.4, http://www.worldclim.org/) data used to in the environmental layers of analysis was an average of information since that year.

MODEL CONSTRUCTION

A maximum entropy algorithm was used to construct distribution models for each of the species of interest. The collected Triatominae occurrence points and bioclimatic environmental layers were used with Maxent software (version 3.3.3k) for a historical average of species distributions across Texas. Maxent was used as it is standard in the literature for use in constructing species distributions from a large number of collected specimens, a presence-only record (10). Bioclimatic parameters were obtained for the state of Texas from WorldClim and are as follows: annual mean temperature, mean diurnal range, isothermality, maximum temperature of the warmest month, minimum temperature of the coldest month, temperature annual range, annual precipitation, precipitation of the wettest month, precipitation of the driest month, precipitation of the wettest quarter, precipitation of the driest quarter, precipitation of the warmest quarter, precipitation of the coldest quarter. The technical specifications of the Maxent run include, a convergence threshold of 1.0 x 10-5, and use of the threshold and hinge features, without duplicates.

FUTURE MODELS

Bioclimatic predictions under the AIM 6.0 pathway, RCP6.0, from the version 1.1 of the Beijing Climate Center Climate System Model (BCC_CSM1.1) GCM were retrieved in a 30 second spatial resolution from WorldClim.

RESULTS

Principle component analysis indicated that of 19 bioclimatic variables, the first two principal components (minimum temperature of the coldest month, and annual mean diurnal temperature for *T. gerstaeckeri*, and precipitation seasonality, followed by precipitation of the coldest quarter for *T. sanguisuga*) account for 44.9% and 47.2% of

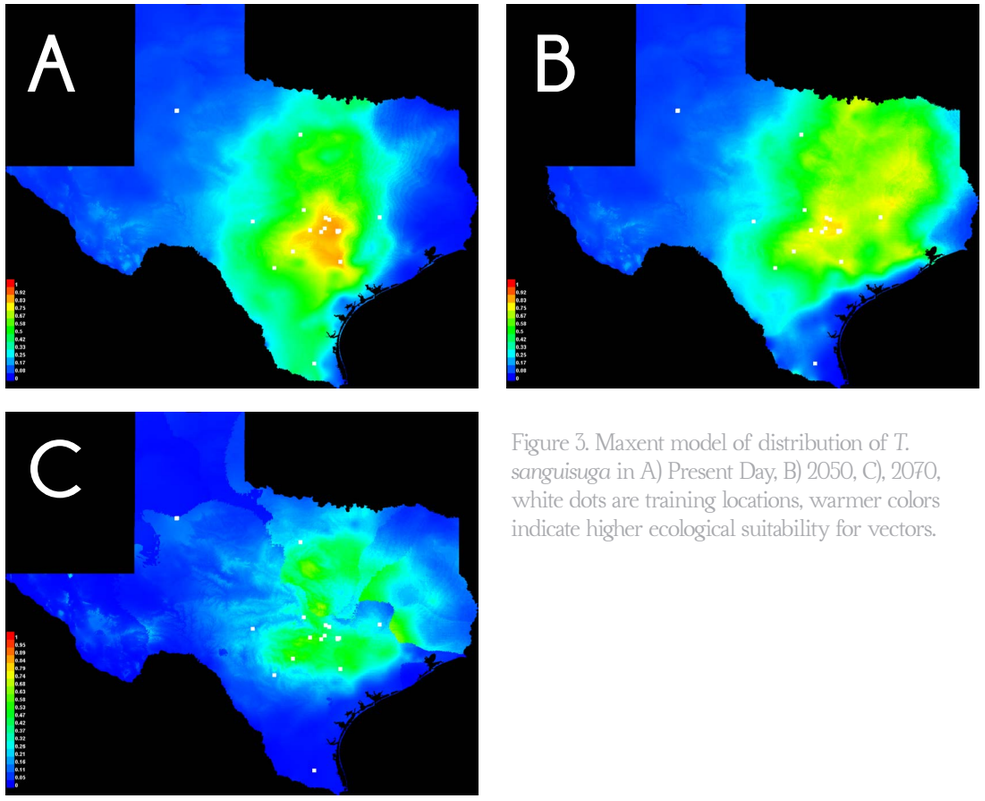

Figure 3. Maxent model of distribution of *T. sanguisuga* in A) Present Day, B) 2050, C), 2070, white dots are training locations, warmer colors indicate higher ecological suitability for vectors.

the explained variance for *T. gerstaeckeri* and *T. sanguisuga*, respectively.

The most important bioclimatic factors for each species (after application of the Jackknife procedure) differed between the two at all time points. For *T. gerstaeckeri*, the most important variable was the minimum temperature of the coldest month, followed by annual mean diurnal temperature, annual precipitation, and precipitation seasonality. For *T. sanguisuga*, the most important predictor variable was the precipitation seasonality, followed by precipitation of the coldest quarter, and minimum temperature of the coldest month. In 2050, the most important variable for *T. gerstaeckeri* changed to annual temperature range. Annual temperature range remained as the most important variable for *T. gerstaeckeri* in 2070.

For *T. gerstaeckeri*, estimates of suitability across the state ranged from 0.00 to 0.92 for the current conditions, between 0.00 and 1.00 for 2050, and between 0.00 and 0.77 for 2070. For *T. sanguisuga*, suitability ranged from 0.00 to 0.85 for the current conditions, between 0.00 and 0.75 for 2050 and between 0.00 and 0.68 for 2070. These both show an overall decrease in suitability. Areas of current high suitability for *T. gerstaeckeri*, such as in South Texas along the USA-Mexico border, saw a decrease in suitability by 85% by 2070. Whereas current areas at medium risk saw a dramatically localized increase of suitability, such as the 46% increase in the Houston and Galveston area, near Trinity bay along the

Gulf coast. Areas of current high suitability for *T. sanguisuga* decreased by 33% by 2070 in central Texas where the highest suitability is currently. No areas saw an increase in suitability for *T. sanguisuga*. Additionally, both species show unchanged low suitability in the Texas panhandle in the northwest part of the state.

DISCUSSION

The current distribution of vectors of Chagas disease is based on bioclimatic variables.

Of the bioclimatic variables studied to determine vector distribution, as listed above, different variables were more important to some species of vector than to others. *Triatoma gerstaeckeri*'s niche was most heavily influenced by the minimum temperature of the coldest month, indicating a threshold temperature, below which the vectors die, most likely while in egg or nymphal stages. The niche of *Triatoma sanguisuga* was determined by the precipitation variation throughout the year, favoring areas with constant and dependable rainfall. This requirement results in a distribution more toward the gulf coast region of Texas and the east of the state, which tend to have more stable precipitation levels than the west and immediately along the coast. (Figure 1A and 1B).

Presence of *T. gerstaeckeri* and *T. sanguisuga* will decrease and shift in response to climate change across the state of Texas.

Under the RCP6.0 climate pathway, "Aim 6.0", the densities of both studied vectors will decrease and the distribution will shift towards the east. By 2050, *T. gerstaeckeri* will have a highly restricted range with an epicenter in central Texas, as well as a large density on the gulf coast near Houston. Both trends are continued in 2070 (Figure 2). In 2070, distribution of *T. gerstaeckeri* is restricted further, with the exception of a large increase of ecological suitability in the southern region, which may be caused by the presence of microclimates along rivers in the area or that the rural area is suited for the vectors than further north in more populous areas (Figure 2). The trends observed for *T. gerstaeckeri* contrasts somewhat with *T. sanguisuga* which will have a much broader range in 2050 and 2070, comparable to its present day range, but with a much lower concentration (Figure 3). The distribution for *T. sanguisuga* will be centered in central and east Texas (Figure 3). The presence of both vectors decreases along the Texas–Mexico border and southernmost regions of the state. This indicates that there is also a maximum suitable temperature being exceeded in these climatic scenarios. The consensus in the field is that Chagas disease rates will increase in America, with Texas being the first state affected. The finding of decreased vector range and density was thus very surprising and did not agree with our hypothesis that vector ranges will increase. However, the vectors studied do not represent all Chagas competent vectors. And while the species studied have been major sources of concern in Texas in the past, they may not be major vectors for disease in the future (14, 20). Both of these species have been endemic to Texas for many years, and are thus likely highly adapted to its current climate (10.1). Future Chagas infections may be caused more by invading species coming from Latin America as the climate warms and becomes too hot to be suitable, and find refuge in Texas with a warmer climate to match that of the previously inhabited endemic areas. Therefore, for a better understanding of risk of infection, further research would open up this study to newer triatomine species to Texas, including: *T. indictiva, T. lecticularia, T. protracta, and T. rubida*. This future direction would look to see if these species show the same northward trend over time. Anthropogenic effects on the environment increase the risk of Chagas transmission.

The risk of Chagas infection increases in cities, specifically in the largest cities of Texas: Houston, Dallas, and Austin due to the high human population in each of the cities. A high human population favors the establishment of a local infection cycle because the triatomine bugs are attracted to areas of human activity and the presence of infected reservoirs increases the risk of transmission to humans (20). While bugs are often found in rural areas, they are attracted to gaseous CO2 which is emitted at higher levels in urban areas than rural ones (23). Larger cities also tend to have high immigration rates; people raised where Chagas is already endemic often are chronically infected without knowing it and can serve as a reservoir to infect naive vectors and perpetuate infection into their new area (4). Larger cities also exhibit an urban heat island, where the inner city is warmer, specifically overnight than the outlying rural or suburban areas (9). This affects the mean diurnal temperature (the average temperature range for each day in any given month), which, as seen with *T. gerstaeckeri*, can have a strong effect on vector distribution (17). Because of this a strong urban heat island, yields a stable overnight temperature, making cities more suitable than other areas for some vectors (20).

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IMATINIB MESYLATE AS AN EFFECTIVE ANTI-VIRAL TREATMENT FOR ALPHAVIRUS INFECTIONS

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• Imatinib

ABSTRACT

Alphaviruses are plus-strand RNA viruses that are transmitted by mosquitoes. There are very limited vaccines and treatment options available to those infected with alphaviruses, resulting in significant human and animal morbidity and mortality each year. Viruses are parasites of host cell metabolism and alphaviruses have been shown to increase glycolytic flux during infection to aid viral replication. Imatinib mesylate is an FDA-approved tyrosine kinase inhibitor that is used to treat several types of cancers. A hallmark of tumorous cells is an elevated metabolic rate and Imatinib successfully slows metabolism by inhibiting tyrosine kinases that are required to activate metabolic enzymes, such as hexokinase in the glycolytic pathway. It was hypothesized that Imatinib could be used to slow metabolism in virally-infected cells and reduce viral replication. Alphavirus-infected cells were treated with various concentrations of Imatinib and at a concentration of 6 μM, viral replication was reduced by more than 40% while cell *via*bility was still at 100%. The efficacy of Imatinib treatment at inhibiting alphavirus replication was confirmed at different times post infection (6, 12, 18, and 24 hours post infection), different levels of infection (multiplicities of infection= 0.1, 1, and 10), and within different cell lines (BHK, Huh7 and HEK). Further analysis in mouse or other animal models is needed to confirm the utility of Imatinib as a therapeutic option for treating alphavirus infection, but the data are promising and shows a significant reduction in viral replication and may represent a novel treatment option for alphavirus infections.

INTRODUCTION

Alphaviruses are enveloped, plus-strand RNA viruses, that are transmitted *via* mosquito vectors. Alphaviruses, including Eastern, Western, and Venezuelan Equine Encephalitis Virus, Chikungunya Virus, Sindbis Virus, and Semliki Forest Virus, are responsible for millions of infections each year (15, 22). Chikungunya virus recently spread to North, South, and CentralAmerica and resulted in more than 2.9 million confirmed and suspected cases, with nearly 300 deaths as of summer 2016 (11, 26). Alphavirus infection results in a wide range of symptoms including rash, malaise, chronic arthralgia, and can even lead to fatal encephalitis (2, 23, 30). There are few effective treatment options available for those infected with alphaviruses and a novel method to treat and inhibit alphavirus infections is imperative to public health.

Traditionally, antiviral therapies have been designed and engineered to target viral proteins, instead of host cell proteins in order to prevent cytotoxicity to the host cells (17). However, due to the rapid rate of mutation and diversity within viral species, viral specific drug treatments are limited in efficacy and burdened with the rapid development of drug resistance. New strategies involve altering or limiting cellular pathways that the virus relies on and thereby reducing the viral replication capacity of the host cell (4). All viruses are parasites of the host cell metabolic pathways and depend on the host cell for all biomolecule and energy synthesis requirements. Many viruses, including alphaviruses, have been shown to manipulate cellular metabolic pathways to enhance the cellular environment and make it optimal for viral replication (8, 9, 19, 29). Sindbis virus (SINV) is the prototype alphavirus and is commonly used as the model virus for other alphaviruses. For Sindbis virus, an elevated glycolytic rate appears to benefit the virus replication(6). It was hypothesized that drugs that inhibit or reduce metabolic rates may effectively be used to slow and inhibit viral replication.

Metabolic inhibitors have been studied extensively in the cancer research fields. Cancer is essentially a metabolic/cell division disorder where cells grow uncontrollably with an elevated metabolic rate. Many cancer drugs target this elevated metabolism and reduce or slow down metabolism to normal levels, thereby inhibiting cancer growth and metastasis (27). Due to the widespread burden of cancer, there has been much research in metabolic inhibitor drugs and there are several options that are FDA-approved and are used clinically to treat cancers (5, 10, 16). Viral infections induce a similar elevated metabolic phenotype in host cells and some of these metabolic inhibitor cancer drugs have been repurposed as novel treatment options for viral infections.

Imatinib mesylate, or Gleevec®, is a tyrosine kinase inhibitor that prevents the phosphorylation and activation of key enzymes in metabolic pathways (1). Specifically, Imatinib has been shown to reduce the activity of hexokinase, which is a key enzyme in the glycolysis pathway (3, 18). Sindbis virus is dependent on glycolysis and it was predicted that inhibiting the glycolytic pathway would result in less viral replication. We hypothesized that Imatinib mesylate, which is already used clinically, may be repurposed and effectively used to treat alphavirus infection. Here we present our findings that Imatinib mesylate successfully reduces Sindbis virus replication in cell culture at low enough concentrations that do not affect host cell *via*bility. Investigating different Imatinib concentrations, viral titers, infection times, treatment addition times, and various cell lines have all shown Imatinib to be efficacious at inhibiting Sindbis virus infection.

METHODS

CELLS AND CELL CULTURE

Baby Hamster Kidney (BHK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with streptomycin/penicillin and 10% FBS. Cells were incubated in T75 flasks or 24-well culture plates and kept in an incubator at 37ºC and 5% CO_2. Cells were passaged every 3 days once 85% confluent using PBS and 0.25% Trypsin. Cells were counted and seeded into well plates (24-well) 24 hours before an experiment so

that the cells would be about 70% confluent at the time of infection/treatment (75,000 BHK cells/ml= ~37,500 BHK cells/well). Human hepatoma (Huh7) cells and Human embryonic kidney (HEK) cells were maintained in similar conditions but were passaged every 4 days.

VIRUS AND VIRAL INFECTION

The 100 +/- 10 mg of fresh rind and curd saSindbis virus, which belongs to *Togaviridae* and is an old world alphavirus, was used as the model alphavirus for all experiments. A Green Fluorescent Protein (GFP) reporter was previously inserted into the viral genome *via* a duplicated subgenomic promoter at the 3' end of the viral genome. This GFP is concurrently expressed with the viral proteins and the relative GFP fluorescence serves as a direct indicator for viral replication in infected cells. These double subgenomic alphavirus reporters are well established and used widely in the alphavirology field(21, 23, 28). Prior to infection, a sample well was used to count cells. Cells were trypsinized, resuspended, and counted using Trypan Blue staining and a hemocytometer. Once an accurate count of cells/well was available, the amount of virus could be calculated and added to the specified wells. Cells were infected with a multiplicity of infection (MOI) of 1, unless otherwise noted. MOI is the ratio of infectious virus particles (plaque forming units (PFU)) to number of cells and an MOI=1 will infect a majority of cells in the well. The corresponding amount of virus was added to the total volume needed and then was added to each well being infected. The plates were incubated for 1 hour at 37ºC to allow the virus to infect and enter the host cells. Following 1 hour of infection, the media containing the virus was removed, the cells were washed with PBS, and fresh media (with or without the treatment) was added to the cells. The infected cells were put back in the incubator and allowed to infect for the specified time.

IMATINIB TREATMENT

Imatinib mesylate was purchased (Sigma) and resuspended in H_2O to make a 100mM stock. This 100mM stock was then serially diluted with two-fold dilutions to acquire stock concentrations at 50mM, 25mM, 12.5mM, 6.25mM, 3.125mM, and 1.56mM. To treat the cells, the mM stocks were diluted 1:1000 in cell culture media to provide media with 100µM, 50µM, 25µM, 12.5µM, 6.25µM, 3.125µM, and 1.56µM concentrations of Imatinib in cell culture media that could be added directly to infected cells. A control treatment was prepared by diluting H_2O solvent 1:1000 in media following the protocol as the drug dilutions. The dilutions of Imatinib in cell culture media were always prepared fresh for experiments. For the experiments with different times of Imatinib addition, the media was removed at the specified time and replaced with fresh DMEM containing the corresponding dilution of Imatinib or the solvent control.

CYTOTOXICITY OF IMATINIB AND PLATE READER

Cell *via*bility was assessed using the alamarBlue Cell *Via*bility Assay® from ThermoFisher according to manufacturer's protocols. Briefly, Imatinib treated cells were stained with a concentration of 100µM Resazurin/alamarBlue and allowed to incubate for 1 hour at 37ºC. Following the one hour incubation, the plates were analyzed on an EnSpire Multimode Plate Reader ® from PerkinElmer for fluorescence at an excitation of 540-570nm and an emission of 580-610nm to measure the *via*ble cells. The mock/control treated cells were set to 100% and then the *via*bility readings from the treated wells were calculated as a percentage of the control. All experiments were performed and analyzed multiple times in triplicate.

QUANTIFYING VIRUS REPLICATION AND FLOW CYTOMETRY

Virus replication was quantified based on the GFP reporter inserted into the viral genome. GFP was measured in infected/treated cells using the plate reader (see above) and an excitation of 488nm and an emission filter of 509nm. Additionally, cells were trypsinized, resuspended, and run over a Guava easyCyte® flow cytometer from Millipore to assess the percent of cells infected (expressing GFP) and the relative levels of GFP fluorescence per cell. 5,000 cells were analyzed for each sample and each condition was run in triplicate. The same parameters, regions, thresholds, and gating was used for all analysis to successfully measure GFP positive cells.

STATISTICAL ANALYSIS

All data graphs were generated with Microsoft Excel. Data and statistical analysis was performed using R Software. Student's t-tests were performed and a *p-value* <0.05 indicated statistically significant differences. Error bars indicate the Standard error of the mean unless noted otherwise.

RESULTS

QUANTIFYING VIRUS REPLICATION AND FLOW CYTOMETRY

Baby Hamster Kidney (BHK) cells were infected with a double subgenomic Sindbis virus (SINV) containing a fluorescent reporter (GFP) protein inserted in the viral genome(21). Virus was added to the cells to achieve a multiplicity of infection (MOI) of one and after one hour of infection, Imatinib was added to specified wells at various low concentrations of Imatinib (0-100µM). After 24 hours of infection, the cells were analyzed for GFP fluorescence as an indicator for SINV replication either with a plate reader or with the flow cytometer. Data from the flow cytometer accurately matched the data from the plate reader, but the flow cytometer allowed more specifics to be analyzed (fluorescence per cell and percent infected). Treatment of SINV-infected BHK cells with Imatinib showed a dose-dependent decrease in viral replication (Figure 1). Cytotoxicity of the Imatinib concentrations was also measured on the BHK cells using alamarBlue Cell *Via*bility Assay. Imatinib started reducing cell *via*bility at concentrations above 12µM, with a dose-dependent curve of cytotoxicity at higher concentrations of Imatinib. Treatment with concentrations less than 12µM resulted in greater than 80% cell *via*bility. However, at 6µM Imatinib treatment, viral replication was significantly reduced by more than 50%. The IC50 and CC50 were calculated to be 4.8µM and 28.1µM, respectively, giving a therapeutic index of 5.85 for using Imatinib as an antiviral for alphaviruses. Ribavirin, which is a well-established antiviral therapy that is used in severe viral infections, has a therapeutic index of around 5-8 for Sindbis virus, indicating that Imatinib treatment is equally effective at treating alphavirus infection(13, 20, 24).

IMATINIB'S EFFECT OVER TIME AND DIFFERENT LEVELS OF SINV INFECTION

Imatinib's effects on SINV virus replication over time was investigated next. Cells were infected with dsSINV-GFP at an MOI=1 and then half of the cells were left untreated and half of the cells were treated with 10µM Imatinib. Cells were analyzed every 6 hours for a 24 hour period to detect GFP fluorescence

and calculate the percentage of cells infected using flow cytometry. Significant inhibition of virus replication (Student's t-test $p<0.05$) was seen in all time points past 12 hours that were treated with Imatinib (Figure 2A). Additionally, BHK cells were infected with an increasing amount of virus and then treated with mock solvent or 10uM Imatinib and allowed to infect for 24 hours. Cells were analyzed on flow cytometry to determine the percentage of cells infected (Figure 2B). Increasing the level of infection (multiplicity of infection= MOI) by up to 10 fold still showed a reduction in virus infection in cells treated with 10μM Imatinib (Figure 2B).

TIME OF TREATMENT AFFECTS IMATINIB INHIBITION OF SINV

We sought to validate Imatinib as a treatment option by investigating different times of treatment and at a higher infection level (MOI=10). BHK cells were treated with 10μM Imatinib at 6 or 3 hours pre infection, at the same time as infection (0hrs), or 3, 6, or 12 hours post infection. The virus was added at time 0 at an MOI=10 and then the cells were allowed to infect for 24 hours before being analyzed on flow cytometry. Each time point was analyzed and the treatment was set as a percentage of the untreated control (Figure 3).

IMATINIB INHIBITION OF SINV WORKS IN MULTIPLE CELL TYPES

To confirm the anti-viral effect of Imatinib on infected cells, the study was expanded to include human cultured cells. Human Hepatoma cells (Huh7) and Human Embryonic Kidney cells (HEK) were utilized by being plated and infected with dsSINV-GFP at an MOI=10 similar to the BHK cells. 10μM of Imatinib treatment successfully reduced SINV replication in both the Huh7 and the HEK cells at 24 hours post infection with an MOI=1(Figure 4).

DISCUSSION

Imatinib mesylate, or Gleevec®, is already FDA-approved and is used regularly in cancer treatments. Clinically, Imatinib is administered orally at low dosages (400mg p.o. b.i.d=.800mg/day)(5). We report that at similarly low concentrations (10 μM), Imatinib reduced SINV replication in cultured cells. This novel finding suggests that metabolic inhibitors used in cancer chemotherapy may be repurposed and used for treating viral infections. Imatinib mesylate works by inhibiting tyrosine kinases within the host cell(5, 18). Many metabolic enzymes require phosphorylation from tyrosine kinases in order to be activated. The general inhibition of tyrosine kinases reduces the ability of the cell to have elevated metabolic rates. This limitation on cellular pathways prevents the virus from manipulating host cell metabolism and hijacking certain pathways for optimal viral replication. During Imatinib treatment, the cell is maintained at lower metabolic rates and therefore limits the ability of the virus to replicate. This hypothesis has been validated and it has been shown that Sindbis virus is significantly inhibited in cells treated with 10μM Imatinib. This concentration of Imatinib was not toxic to the cells and presents a therapeutic window where Imatinib may be used effectively to limit virus replication, while maintaining host cell *via*bility (Figure 1). Imatinib's therapeutic index was similar to Ribavirin, which is a clinical antiviral used to treat severe cases of Respiratory Syncytia virus (RSV)(12). Although it would be more advantageous if Imatinib had a higher therapeutic index, the proof-of-principle is still validated that anti-cancer and anti-metabolism compounds may be repurposed and used for antivirals. Imatinib is effective against Sindbis virus and investigation of other anti-metabolism compounds may lead to the discovery of an even more effective drug that can be used to treat viral infections.

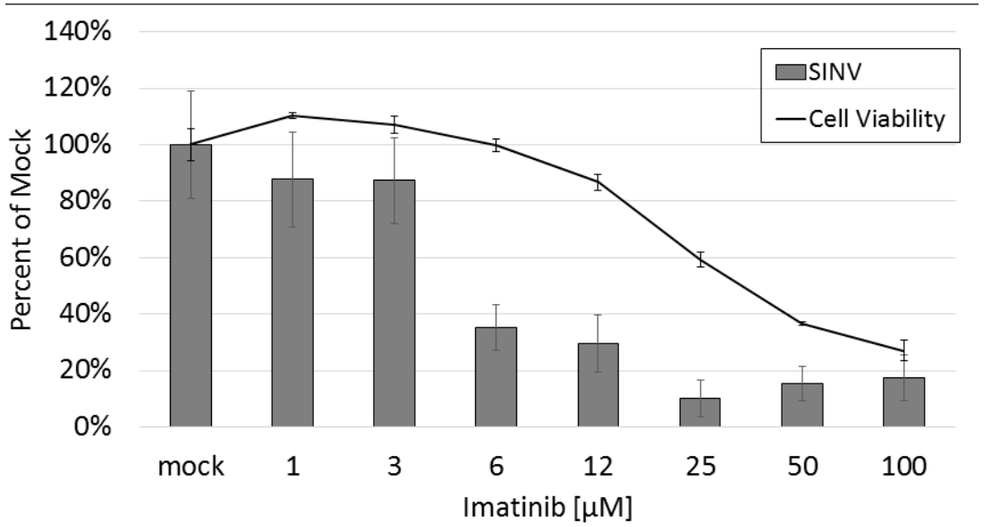

Figure 1: Imatinib inhibition of Sindbis virus and cell *via*bility
Flow cytometry analysis of BHK cells infected with dsSINV-GFP at an MOI=1 and treated with dilutions of Imatinib SINV replication (Gray bars) and cell *via*bility (Black line) are shown with increasing concentrations of Imatinib. Both the mock/untreated samples were set to 100% and the different treatment conditions were calculated as a percentage of the mock. SINV was significantly inhibited at 6 μM of Imatinib compared to mock treated ($p<0.05$). Error bars represent standard de*via*tion n=3.

This Imatinib anti-viral effect was confirmed at different times during the virus replication cycle and indicates a general overall reduction in virus infection as compared to a step/cycle specific inhibition effect. Over the course of 24 hours, untreated cells showed a gradual increase in percent of SINV- infected cells eventually reaching about 25% infected, which is statistically significant when compared to the Imatinib treated cells that only reached about 5% of cells infected at 24 hours. Significant reduction in virus replication was seen in a wide variety of infection levels, as indicated by different infection MOI's (Figure 2). Interestingly, the plates that were examined at different time points showed higher infection levels (up to 25%) compared to the MOI plates (only up to about 17%). This may be due to the fact that the time course plates were physically moved every 6 hours, which would result in a disruption of the media and may have redistributed the virus supernatant throughout the culture. These results imply that Imatinib may be successfully used to reduce viral loads at different times and levels of infection.

To further assess the efficacy of Imatinib to lower viral replication, the impact of treatment timing on viral infection was investigated. Understanding the opportune timing for treatment is a critical component for antivirals to discover if the compound is more effective if given prophylactically prior to exposure or if post-infection treatment is more effective. The greatest inhibition of virus replication was seen in cells that received the Imatinib treatment 3 or 6 hours prior to infection. Adding the Imatinib at the same time as infection or up to 3 hours post infection still achieved about a 20% reduction in virus replication, but adding

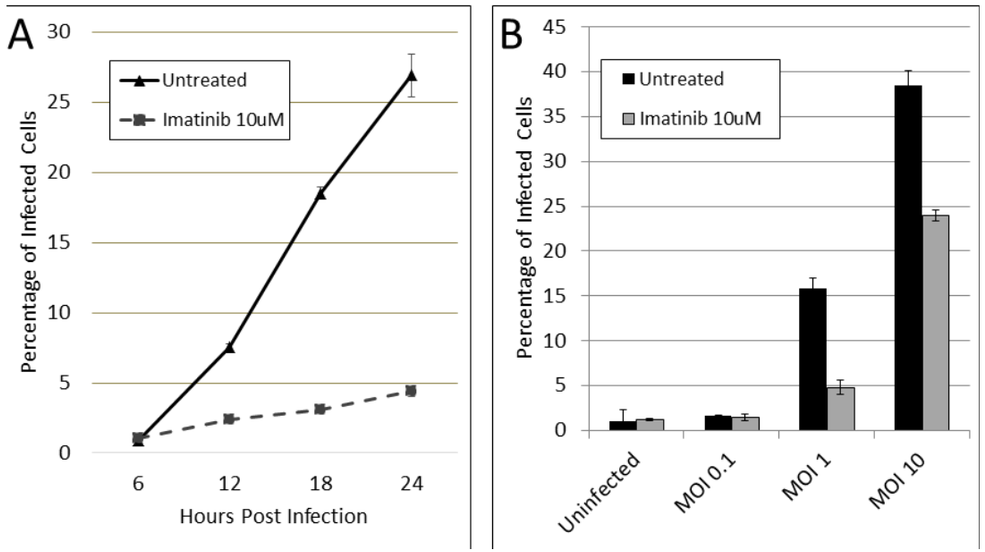

Figure 2: Imatinib treatment at different times post-infection and SINV concentrations
A) SINV-infected BHK cells (MOI=1) were treated with 10μM Imatinib (dotted line) or left untreated (black line) and analyzed every 6 hours for the percentage of cells infected using flow cytometry. B) BHK cells infected with increasing amounts of virus and treated with 10 μM Imatinib (gray) or left untreated (black). The percentage of cells infected was determined with flow cytometry at 24 hours. Significant reductions (Students T-test P<0.05) in dsSINV-GFP replication are indicated with an asterisk (*).

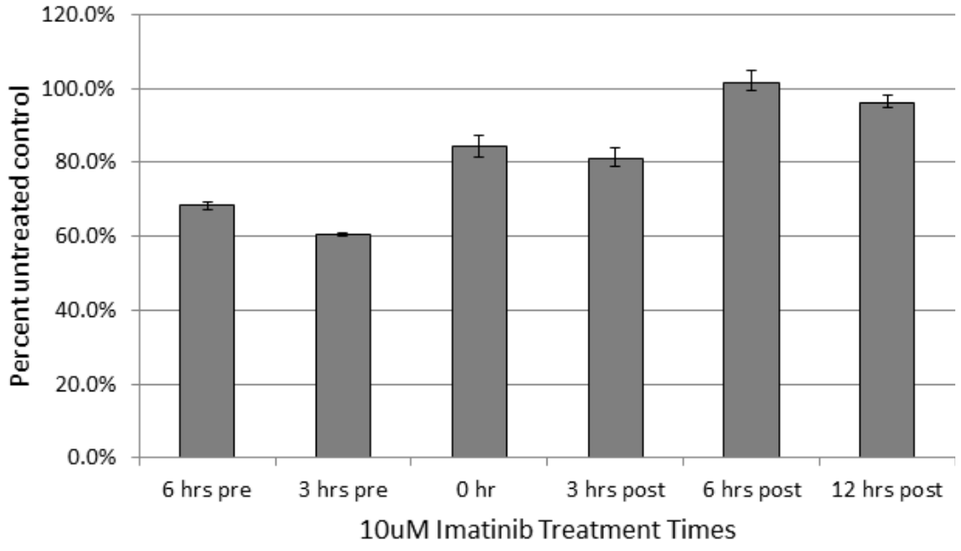

Figure 3: Treatment with Imatinib at different treatment times
BHK cells were treated with 10 μM Imatinib or solvent control at the times indicated and dsSINV-GFP (MOI=10) was added at time 0. At 24 hours post infection, the cells were analyzed for dsSINV-GFP *via* flow cytometry. The untreated sample for each condition was set to 100%. The greatest difference was seen with a single treatment.

the Imatinib 6 hours or later after infection did not result in a significant inhibition of virus infection (Figure 3). The 20% reduction is not as significant as what was seen in previous experiments, but that is likely due to the higher MOI and also greater movement of the plate during the infection incubation. Pre-infection treatment with Imatinib reduced the virus replication more effectively than treatments that were added concurrently or post infection. This indicates that Imatinib may be most effective as a prophylactic drug, given prior to high risk exposure situations. However, due to the inherent side effects of anti-cancer and anti-metabolic drugs, prophylactic treatment might not be feasible in a clinical setting, but post-infection treatment may be effective. Although not as significant as a pre-infection treatment, even treating with Imatinib at 3 hours post-infection successfully reduced virus infection and confirms the potential utility of using Imatinib as a post-exposure/infection treatment option (Figure 3).

The Imatinib inhibition of SINV infection was further confirmed in human cultured cells, both liver (Huh7) and kidney cells (HEK), which both showed a similar reduction in virus replication as was observed in the BHK cells (Figure 4). Huh7 and HEK cells are common cell lines used in virology to assess virus replication in human tissue and it is promising that both cell lines show an effective reduction in SINV replication when treated with 10μM Imatinib. Variation in viral reduction is likely due to specific differences in the cell type, but the overall trend of viral inhibition with Imatinib treatment is maintained in the different human cell lines. This validates the potential efficacy of using Imatinib as a therapeutic anti-viral treatment option in human alphavirus infections.

In this study, a single metabolic inhibiting cancer drug known as Imatinib mesylate was investigated. Imatinib has shown very promising inhibition of Sindbis virus replication at different times, levels/MOI's, and host cells. Further work will be done

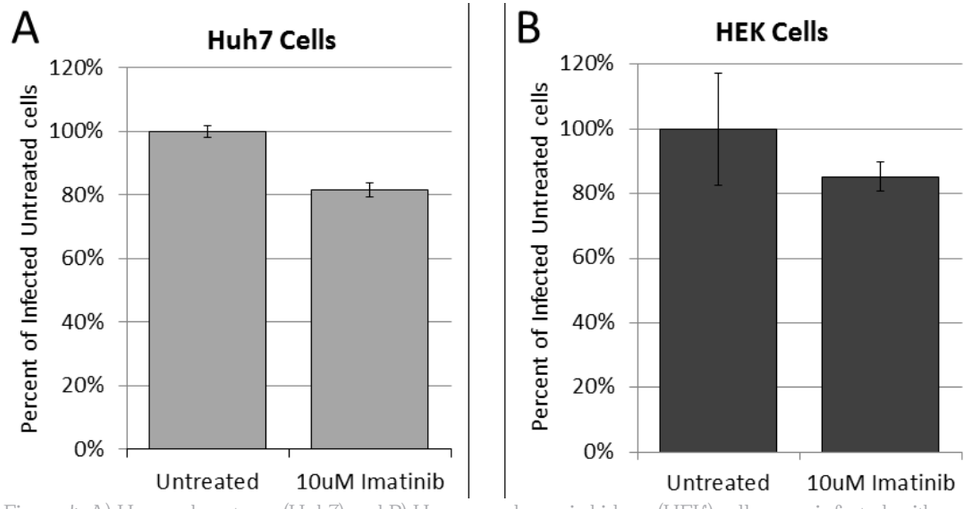

Figure 4: A) Human hepatoma (Huh7) and B) Human embryonic kidney (HEK) cells were infected with dsSINV-GFP at an MOI=10 and then treated with 10 μM Imatinib or solvent control (untreated). At 24 hours post infection, the cells were analyzed for dsSINV-GFP *via* flow cytometry. All data is displayed as a percentage of the untreated samples. (n=6).

to validate and confirm these findings with Imatinib in model organisms and in primary cells. Additionally, there are many other FDA-approved cancer drugs that specifically target and slow cellular metabolism(7, 14, 25, 27). Further work will be performed to screen other metabolic inhibitor compounds as effective antivirals. By studying clinically used and already approved compounds, it may be possible to discover a novel antiviral treatment option that can rapidly be used to treat and bring aid to the millions of alphavirus infections that are occurring globally.

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ORGANIZATION OF THE MULTIGENE FAMILIES OF AFRICAN SWINE FEVER VIRUS

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KEYWORDS

- African swine fever virus
- Multigene family
- Annotation
- Bioinformatics
- Genomics

ABSTRACT

African swine fever virus is a complex DNA virus that infects swine and is spread by ticks. Mortality rates in domestic pigs are very high and the virus is a significant threat to pork farming. The genomes of 16 viruses have been sequenced completely, but these represent only a few of the 23 genotypes. The viral genome is unusual in that it contains 5 multigene families, each of which contain 3–19 duplicated copies (paralogs). There is significant sequence divergence between the paralogs in a single virus and between the orthologs in the different viral genomes. This, together with the fact that in most of the multigene families there are numerous gene indels that create truncations and fusions, makes annotation of these regions very difficult; it has led to inconsistent annotation of the 16 viral genomes. In this project, we have created multiple sequence alignments for each of the multigene families and have produced gene maps to help researchers more easily understand the organization of the multigene families among the different viruses. These gene maps will help researchers ascertain which members of the multigene families are present in each of the viruses. This is critical because some of the multigene families are known to be associated with virus virulence.

INTRODUCTION

African Swine Fever Virus (ASFV) is a large dsDNA virus in the family *Asfarviridae*; 23 genotypes have been characterized by sequencing of the p72 gene (1, 12, 13). The virus is endemic in many regions of Africa where it infects primarily warthogs and is spread *via* the bites of soft ticks (9). Although ASFV causes mild symptoms in warthogs and produces no symptoms while replicating in the ticks, it causes very serious haemorrhagic disease in domestic pigs and wild boar. In these animals, the mortality rate approaches 100% for some ASFV strains (18). The relatively recent (2014) but extensive spread of ASFV through Africa to parts of Central Europe takes a significant toll on both small and large-scale pig farming operations in these regions, putting a large strain on the global pig trade (5).

To date, most successful viral prevention methods rely on routine degenerate PCR screening of wild pig and tick populations together with a rapid and competent diagnosis program when an outbreak is suspected. In addition, strict sanitary control procedures must be implemented to reduce the possibility of infected wild hosts interacting with domestic pigs (7). When outbreaks occur, currently, the only effective response is culling of an infected herd and the imposition of a ban on the movement of adjacent herds (2). This produces serious economic problems for the farmers and may incentivize noncompliance. Clearly, an unhindered pork trade would be very beneficial and benefit a large proportion of the population. Between 2014 and 2015, close to $55 million was spent on ASFV prevention in the Baltic States alone, which was considered to have prevented US$4.5 billion in potential losses (7).

Sixteen full ASFV genomes have been sequenced to date, and more than 100 will be sequenced in the next 2–3 years (E. Okoth, personal communication). The availability of these genome sequences is important because comparative genomics analyses will allow researchers to better correlate gene content and amino acid sequence variation with virulence and antigenic variation. However, all ASFV isolates have at least 5 multigene families (MGF) that are made up of sets of paralogs, which are frequently but not always arranged in tandem. Not only do the different viruses have different numbers of these paralogs, but they frequently have indels that remove multiple genes and partial genes resulting in some gene fusions (4,6,14). Consequently, when these viral genomes are aligned by software tools these regions are not aligned correctly. The problem is made more complicated by the fact that the individual MGFs have sometimes been mis-annotated due to failure to identify the correct ortholog groups within the sets of paralogs. Correct identification of the members of these MGFs is especially important because these genes have been linked with virus virulence (6).

The challenge of developing an effective vaccine stems, in part, from the high antigenic diversity distributed among the different strains of ASFV and therefore from the genomic variation. Although it is possible to induce immunity in pigs that protects from challenge with a homologous genotype, the

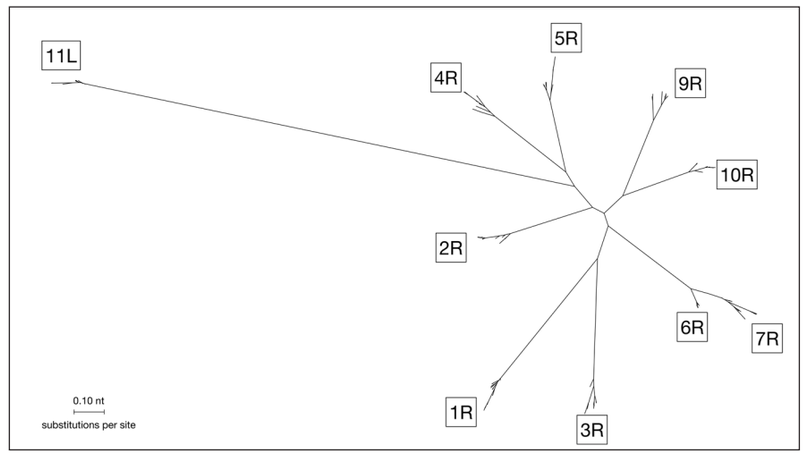


Figure 1. Maximum likelihood phylogenetic tree of MGF 505 DNA sequences was created with RAxML using the GTRGAMMA base substitution model. Sequences were aligned with MAFFT and trees visualized using MEGA7.

MGF	Paralogs	Size (bp)	Conservation	Mis-annotations
MGF 100	3	375-440	High	1
MGF 110	13	315-875	Low	27
MGF 300	3	315-800	High	5
MGF 360	19	960-1100	Moderate	17
MGF 505	10	1500-1630	Moderate	12

Table 1. Summary of the 5 MGFs for the 16 ASFV strains. The relative conservation between the paralogs inversely correlates with the number of paralogs. The "Mis-annotations" column indicates the number of

generation of protection against a heterologous genotype has proved unreliable (15). In fact, vaccination does not always adequately protect against viruses of the same genotype (19).

Here we describe the reannotation of the ASFV MGFs using a common nomenclature that will facilitate future ASFV genome comparisons and provide clarity for the discussion of the differences between viral gene sets.

METHODS

DATA SET

Genomes of the following ASFV isolates were used, GenBank accession numbers are given in parentheses: ASFV-Benin_97_1 (AM712239); ASFV-L60 (KM262844); ASFV-E75 (FN557520); ASFV-OURT_88_3 (AM712240); ASFV-NHV (KM262845); ASFV-Mkuzi_1979 (AY261362); ASFV-BA71V (U18466); ASFV-Georgia_2007/1 (FR682468); ASFV-Pretorisuskop_96_4 (AY261363); ASFV-Warmbaths (AY261365); ASFV-Warthog (AY261366); ASFV-Tengani62 (AY261364); ASFV-MWI_Lil_20_1_1983 (AY261361); ASFV-Ken05_Tk1 (KM111294); ASFV-Ken06 (KM111295); ASFV-KEN_1950 (AY261360).

PHYLOGENETIC TREE AND DOTPLOT CONSTRUCTION

A multiple sequence alignment (MSA) of the 16 complete ASFV genomes was generated using MAFFT (10). Base-By-Base (BBB; (8)) was used to visualize the MSA and highlight the differences between the genomes. Maximum-likelihood phylogenetic trees were constructed using RAxML (16) under the GTRGAMMA base substitution model using 1000 bootstrap replicates. MEGA7 (11) was used to visualize and manipulate the phylogenetic tree output.

Since alignment tools such as MAFFT treat genomes as linear syntenic sequences, they are unable to accurately display any sequence transpositions. Similarly, it can be difficult to assess small differences in the quality of the various possible alignments for the ASFV MGFs from a MSA. Therefore dotplots, which provide a 2-dimensional visualization of all nucleotides-against-all nucleotides were used to supplement genome alignments (JDotter; (3)). A dotplot was created for each individual MGF gene compared against a full length ASFV reference genome. The series of matrix alignments across the dotplots created a unique "barcode" describing the relationship of the gene to all the paralogs in the MGFs. The dotplots were especially useful for determining the breakpoints between fused paralogs.

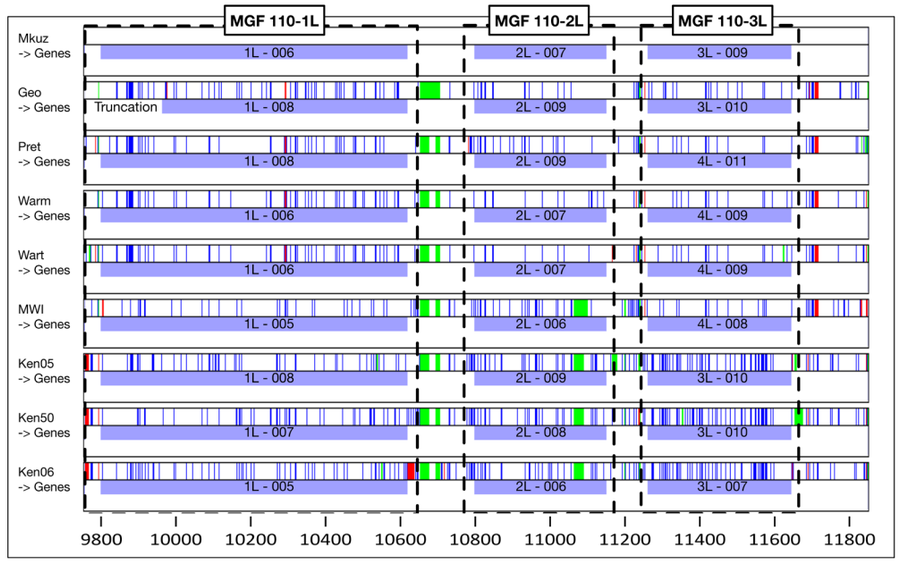


Figure 2. Visual summary from BBB of MGF 110-1L/-2L/-3L paralogs from 9 ASFV genomes. Thin vertical dark blue bars, red and green blocks represent SNPs and insertions and deletions with respect to the topmost sequence. Light blue blocks represent ORFs. Gene labels, positioned on the ORFs, indicate previous annotations (note inconsistencies) as well as gene number. Boxed gene labels indicate new nomenclature.

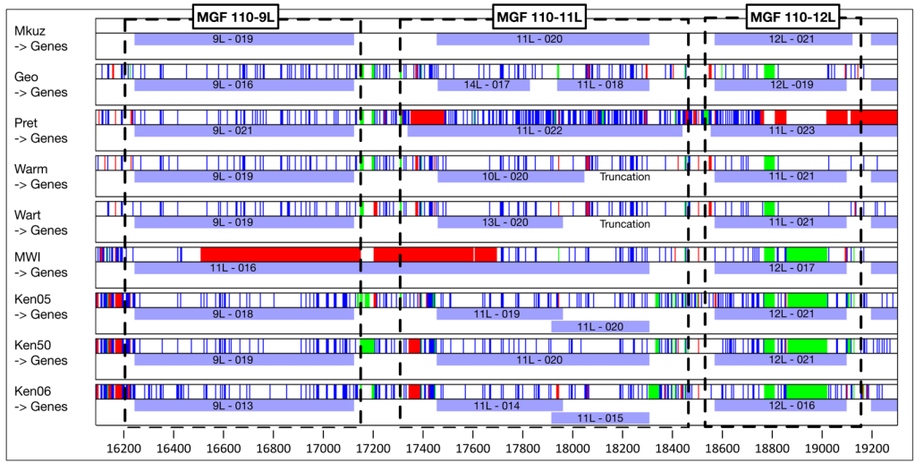


Figure 3. Visual summary displaying fragmentation of MGF 110-11L orthologs. Gene labels indicate the previously annotated orthologs. Thin vertical dark blue bars, red and green blocks represent SNPs and insertions and deletions with respect to the topmost sequence. The MGF 110-10L is not represented in this diagram due to annotation only in Warmbaths strain that is more likely a truncated MGF 110-11L ortholog. Nucleotide positions are mapped at the bottom of the figure.

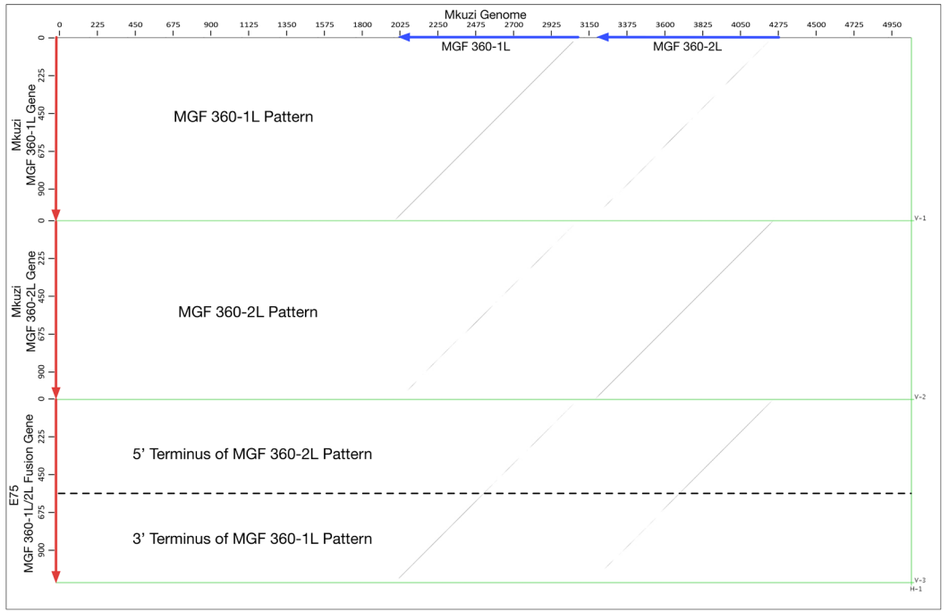


Figure 4. Dotplot of MGF-360 genes. The horizontal sequence contains ASFV-Mkuzi MGF-360-1L and MGF-360-2L genes (blue arrows). The vertical axis represents 3 sequences; the top 2 panels are the ASFV-Mkuzi MGF-360-1L and MGF-360-2L genes as controls and the bottom panel represents the ASFV-E75 MGF-360-1L/2L fusion (red arrow). The dashed line in the bottom panel separates the 5' half of 360-2L and the 3' half of 360-1L. Self-plots of genes generate a solid diagonal black line whereas plots of paralogs produce fainter intermittent lines.

CONSTRUCTION OF MGF MAPS

The MGF maps were created as vector graphics with Omnigraffle (Omni Group, Seattle) on iMac computers. These diagrams can be fully edited to incorporate new genomes and new MGF orthologs as they are discovered.

RESULTS

The goal of this analysis was to create an accurate reference map of the distribution of MGF members throughout the 16 sequenced ASFV genomes. Since the MGF members are not simply present or absent, an annotation scheme was also required to describe the various gene fragmentation/truncation/fusion patterns that exist in the different virus strains. Since we do not yet know the functional consequences of these multiple rearrangements on the biology of the viruses, the purpose of the map is primarily to flag the various differences that exist between the ASFV MGFs. In addition, due to the extremely complex nature of the indels in the MGF regions, which compound when MSAs are generated, we opted to illustrate general variations in the open reading frame (ORF) patterns rather than try to capture every single difference. Our results are sufficient to flag differences between the paralogs so that a detailed DNA sequence alignment of the region can be performed if more information is required for a particular study.

There are currently 5 known MGF series observed in ASFV. Paralogs within an MGF series are numbered chronologically as they appear in the ASFV genome and are classified

Figure 5. Example of summaries showing organization of MGF-505. The ASFV strain is given in the red boxes at the left. Paralog names are shown in the boxes at the top of the diagram along with the size of the ortholog from the reference genome, Mkuzi. The annotation of each gene (arrow head indicates direction of transcription) is in two parts: the paralog group followed by the gene number of the virus strain. If the paralog annotation is incorrect, it is labelled in red. The gene box size represents the relative gene size of orthologs, but is not to scale, nor does it reflect the size relationship between paralogs. Genes connected by grey boxes indicate the fusion of two orthologs.

as "R" or "L" indicating that this gene is either transcribed on the forward or reverse strand respectively.

Our first step in reviewing the relationships between the paralogs/orthologs of each MGF was to create a phylogenetic tree. The MSA was generated using MAFFT with the DNA sequences and the phylogenetic trees were constructed with RAxML. Figure 1 illustrates the value of the trees by showing a visual representation of the relationship between the paralogs of the MGF-505 series (MGF average size 505 amino acids). For example, the tree shows that paralogs MGF-505-6R and MGF-505-7R result from a relatively recent duplication. However, it must be appreciated that phylogenetic trees also hide the raw data, which may have valuable information about the sequences. For example, recombination events and deletions that fuse two paralogs are likely to be lost if only the tree is viewed. Therefore, to ensure that the tree generation step was not flawed by faulty input data, we checked the MSAs with BBB, a MSA editor that also provides highlighting of the differences between pairs of sequences in the MSAs. This helps the researcher recognize MSA regions that have inconsistent similarity levels and may be the result of gene fusion events.

In addition to displaying sequence alignments, BBB is capable of generating a "summary view" of sequence alignments, that captures the positions of SNPs and indel information to allow large MSAs to be shown on a single page. Figures 2 and 3 illustrate the use of BBB to view parts of MGF-110. A truncation of ASFV-Geo MGF-110-1L is shown together with previous alternate naming of ASFV MGF-110-3L orthologs (Figure 2). In MGF-110, we propose 13 paralogs whereas 14 had been previously annotated among the 16 genomes because fragments of a single gene had been annotated separately. Figure 3 shows several of the difficulties faced

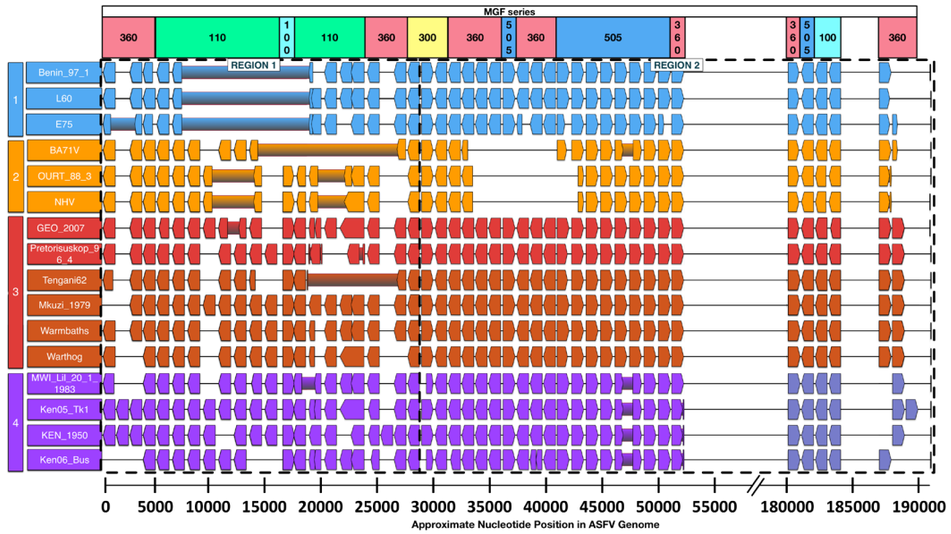


Figure 6. Display of all MGF paralogs as they appear, left to right, in the 16 ASFV strains. Columns of boxes represent individual MGF orthologs as in Figure 5. Location of the different MGF series is annotated above the diagram. The arrangement of MGF orthologs separates the genomes (coloured rows) into 4 groups that correlate well with the phylogenetic tree. It is notable that the MGF compilation can be split into 2 regions, between MGF 300-1L and MGF 300-2R, reflecting significantly more variation in region1 than 2. Nucleotide positions are given at the bottom of the figure.

in trying to annotate the MGFs consistently: 1) MGF-110-11L is fragmented in several viruses, 2) A large deletion in ASFV-MWI creates a fusion of MGF-110-9L/11L, and 3) Large indels (red and green blocks, which illustrate insertion or deletion with respect to the reference) create orthologs of significantly different sizes (MGF-110-12L). Since the ORFs are displayed by BBB across gapped alignments, they are not accurate representations of their true size.

Although MSAs do show raw data (the actual aligned DNA sequences), because they display a one-dimensional representation of the alignment they are of less use when regions of sequences may have been rearranged. In such situations, the two-dimensional presentation of global sequence comparisons from a dotplot can better show rearrangements. For example, Figure 4 shows the comparison of the ASFV-E75 MGF-360-1L/2L fusion with the 2 parental orthologs. It also shows the results of paralog comparison (1L and 2L for ASFV Mkuzi) and ortholog comparison (1L for ASFV-E75 and ASFV-Mkuzi).

After reviewing data from phylogenetic trees, MSAs and dotplots, we constructed a summary diagram for each MGF. These are presented as Supplementary Figures 1-6, which are provided at a large scale to present much greater detail . For each summary diagram, there is also an "information sheet" that explains the representations of the MGFs (Supplementary Figures 7-12). These figures are also available from the Viral Bioinformatics Resource Center, in the ASFV section (https://virology.uvic.ca/organisms/dsdna-viruses/asfarviridae/). Figure 5 is an example of one of the summary diagrams and Table 1 shows the varying complexity of the individual MGFs.

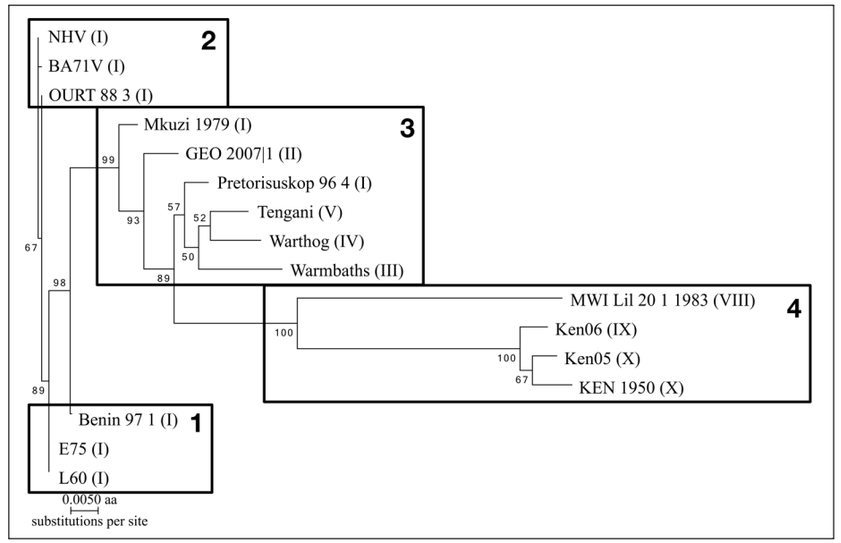


Figure 7. Phylogenetic tree for the 16 ASFV strains. Genotypes are shown in parentheses. Boxes indicate groupings defined in Figure 6. MAFFT was used to align the concatenated amino acid sequences of phosphoprotein p32, structural proteins p72 and p54, and the chaperone protein B602L. The tree was created with RAxML under a GTRGAMMA base substitution model using 1000 bootstrap replicates and visualized using MEGA7. This phylogenetic tree places ASFV strains into clades that replicate the groupings generated by overall MGF gene characteristics.

Although many of the MGFs are similar among the different ASFV strains, there are some differences that are specific to particular clades of the ASFVs. Examples of these are shown in Figure 6 with a full genome phylogenetic tree provided in Figure 7. From these figures, it is clear that the MGFs are relatively fluid, with differences appearing even between ASFV-E75 and –L60, which are very similar. However, some of this variation is expected given the overall variation between the ASFV strains. Interestingly, although these viruses are all denoted as ASFV strains, there is significant divergence between them. A comparison of the ASFV B602L, p32, p54, and p72 genes (as concatenated amino acid sequences) of the 16 ASFVs revealed that there are several viruses that have diverged to be 94 – 95 % identical (aa). In contrast, we found that poxviruses, which belong to a different family of large DNA viruses, that are classified as separate species within the Capripox or Orthopox genera may be 97 – 99 % identitical (aa) in pairwise alignments. Thus, ASFVs that are currently classified as different genotypes within a single species may well be classified into different species if taxonomic standards that are used with poxviruses were applied to ASFV.

DISCUSSION

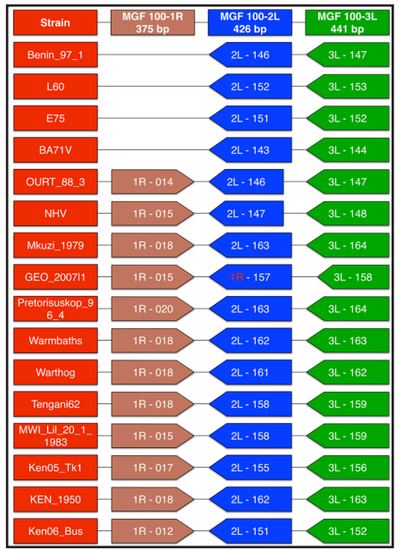
The genomic regions that encode the 5 MGFs of ASFV presented here are highly variable and are hotspots for indels making both sequence alignment and accurate annotation difficult. This has resulted in inconsistent annotation among the 16 ASFV genomes for the identification of paralogs and especially the naming of gene fusions. Since

a large number of ASFV genomes will be sequenced in the near future, we decided that standardizing the annotation and presentation of the ASFV MGFs would greatly simplify genome annotation in the future. With a better reference system for the ASFV MGFs available, we envision a 3-part process in the annotation process for ASFV genomes sequenced in the future. First, there would be a basic sequence similarity search with a set of 10 conserved genes to identify the most similar reference genome to be used with the Genome Annotation Transfer Utility tool (GATU; (17)). Second, a dotplot would be used to confirm co-linearity between the proposed reference genome and the newly sequenced target genome. Third, GATU would be used to transfer as many annotations as possible (> 95 %) from the reference genome to the target, with the use of a full genome alignment of the reference and target in BBB to confirm the positions and numbering of the members of the MGFs. As the number of sequenced and annotated genomes increases, fewer differences will be found between the new target genomes and their references. Thus, GATU will become more efficient and less annotator intervention will be required to annotate those few ORFs that GATU leaves unfinished.

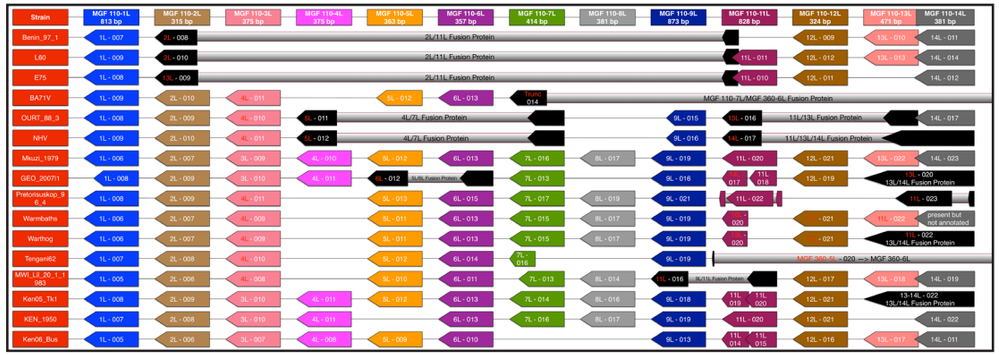
In conclusion, we believe that our figures are an intuitive visualization of the arrangement of genes within the MGFs, especially when there is a need to compare the MGFs of different viruses. It is envisioned that the maps of the ASFV MGFs will be living documents, updated, by a volunteer curator from the research community, with any new paralogs that may be discovered in newly sequenced genomes. This is likely to be required since genomes have yet to be sequenced from a large proportion of the ASFV genotypes and the MGFs are the most variable parts of the genomes. To this end, the diagrams can be easily updated when new MGF paralogs are discovered by the addition of new columns. Although new ASFV genomes can be added to the diagrams by simply copying the most similar existing row and editing the sizes of the gene boxes and labels, as the number of genomes grows, space could be saved by showing a single representative if multiple viruses have identical MGFs.

These MGF maps will speed up the annotation process and simplify the comparison of new ASFV genomes. With more accurate genome alignments, researchers will also be better able to correlate genomic features with virulence levels of the various ASFV isolates.

SUPPLEMENTAL FIGURES



Supp. Figure 1. Diagram of MGF 100 organization. See Supp. Figure 7 for additional information describing this MGF.

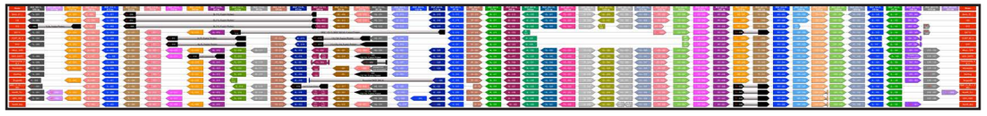


Supp. Figure 2. Diagram of MGF 110 organization. See Supp. Figure 8 for additional information describing this MGF.

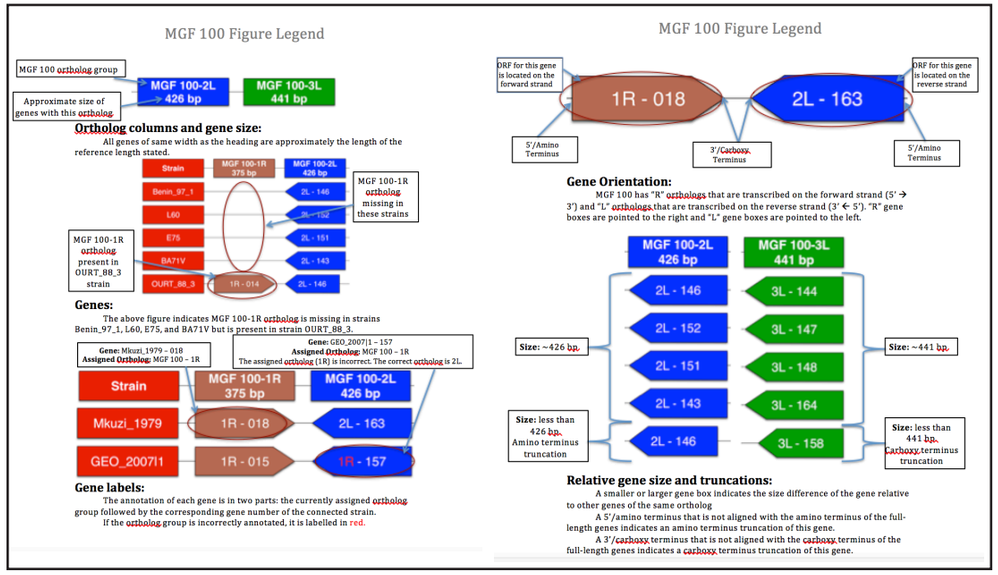
Supp. Figure 3. Diagram of MGF 300 organization. See Supp. Figure 9 for additional information describing this MGF.

Supp. Figure 4. Diagram of MGF 360 organization. See Supp. Figure 10 for additional information describing this MGF.

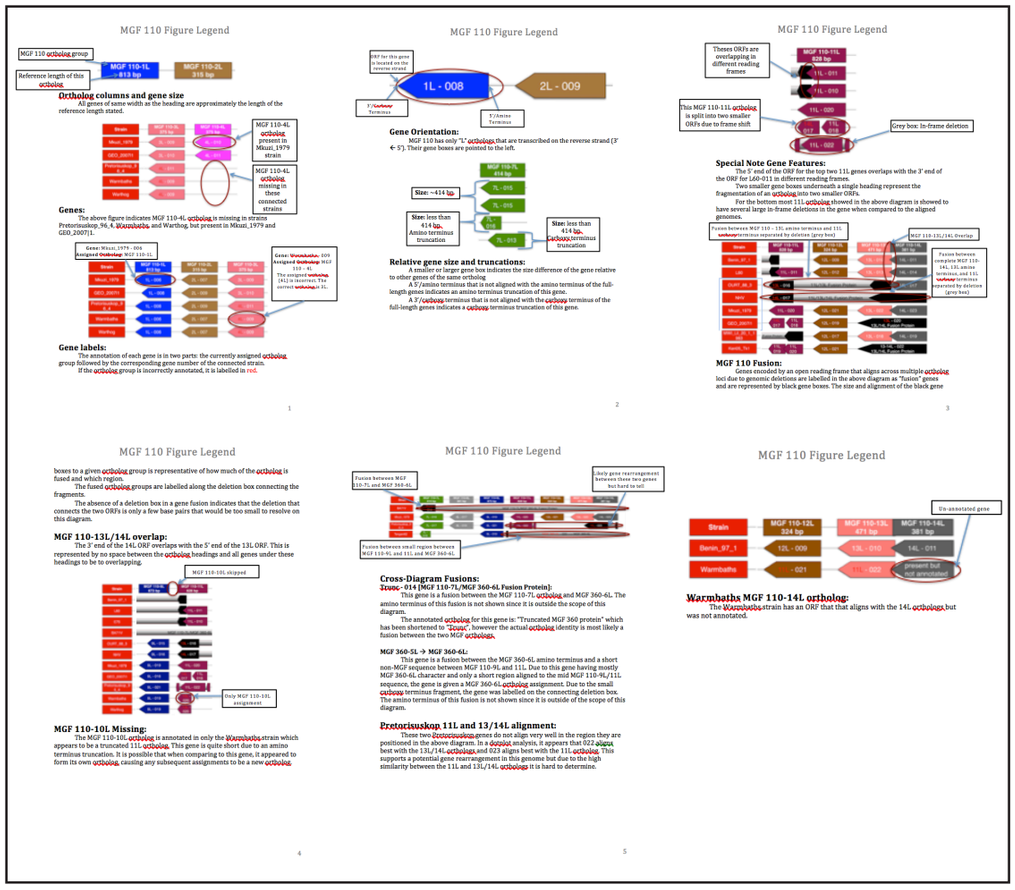
Supp. Figure 5. Diagram of MGF 505 organization. See Supp. Figure 11 for additional information describing this MGF.



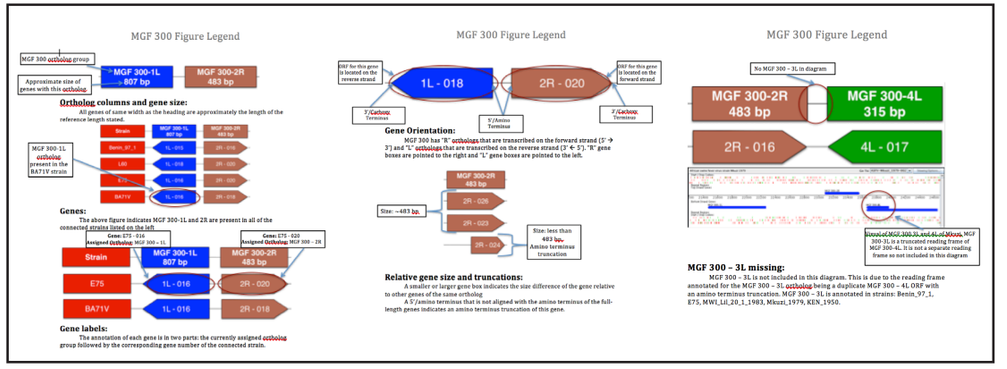
Supp. Figure 6. Diagram of MGF compilation. See Supp. Figure 12 for additional information describing this MGF compilation.



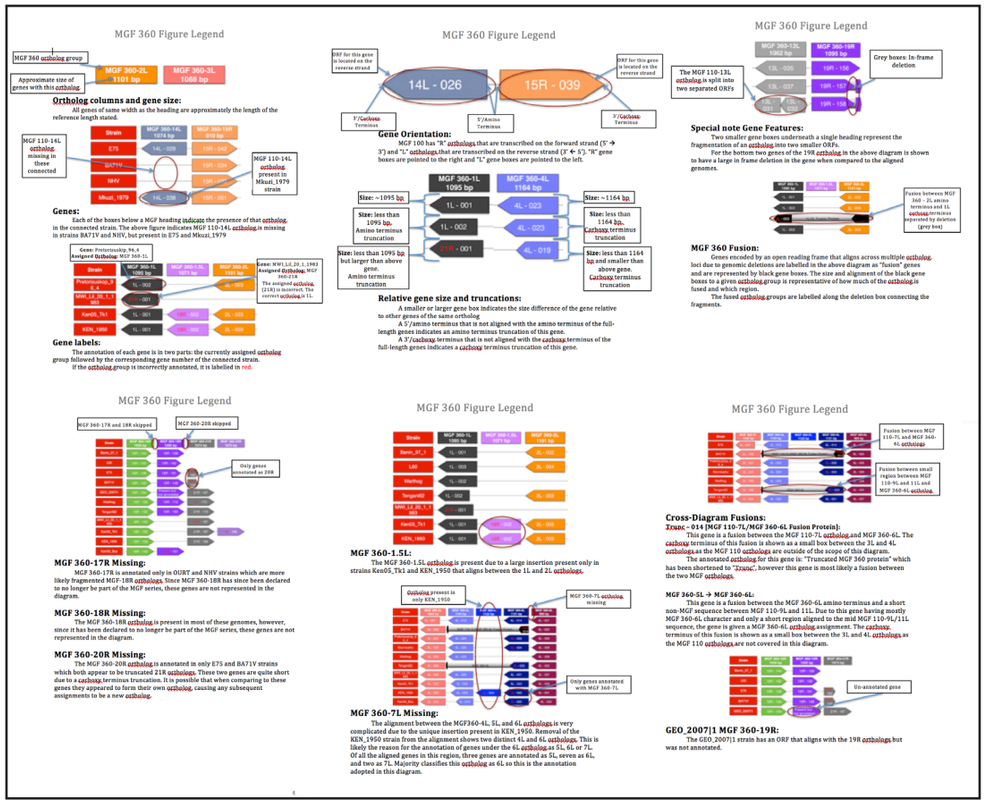
Supp. Figure 7. Information sheet describing MGF 100.



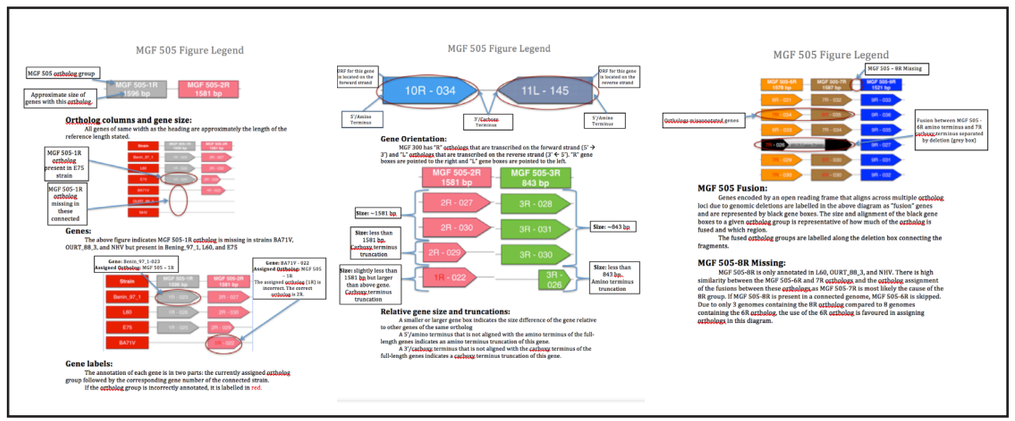
Supp. Figure 8. Information sheet describing MGF 110



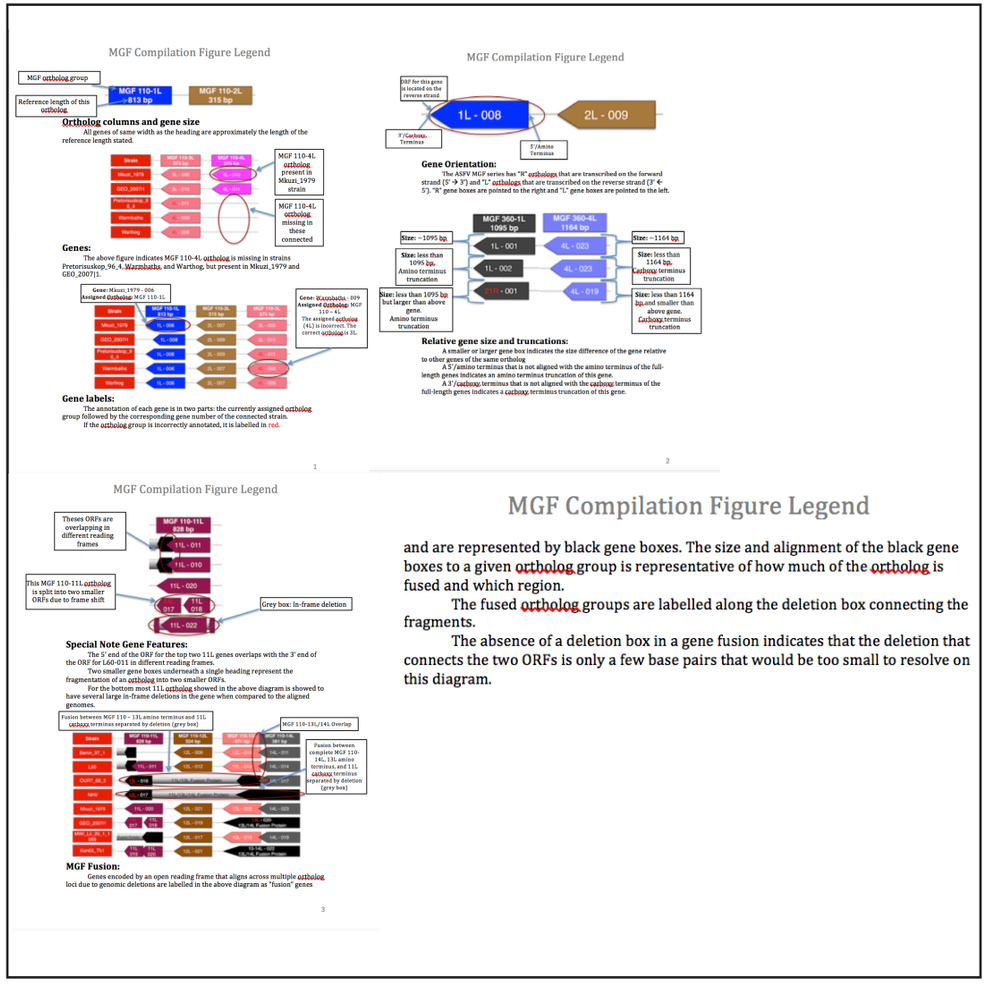
Supp. Figure 9. Information sheet describing MGF 300.



Supp. Figure 10. Information sheet describing MGF 360.



Supp. Figure 11. Information sheet describing MGF 505.



MGF Compilation Figure Legend

and are represented by black gene boxes. The size and alignment of the black gene boxes to a given ortholog group is representative of how much of the ortholog is fused and which region.

The fused ortholog groups are labelled along the deletion box connecting the fragments.

The absence of a deletion box in a gene fusion indicates that the deletion that connects the two ORFs is only a few base pairs that would be too small to resolve on this diagram.

Supp. Figure 12. Information sheet describing MGF compilation.

ACKNOWLEDGMENTS

We would like to thank Chad Smithson for his help and the many Co-op students from the University of Victoria who have built the Viral Bioinformatics Resource Center (virology.uvic.ca).

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UNDERGRADUATE PERSPECTIVE : THE GRANT APPLICATION PROCESS

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CURRENT ADDRESS:
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SUMMATION

The requirement for monetary aid has been needed in scientific research since Galileo and prior. Grant writing is an art form that scientists use in order to move their research along, regardless of the size of the award. One of the largest struggles is the ability to write the technical piece, describing the research being performed, the need for money, and what it will be used for. Writing the piece in lay terminology can be one of the most challenging experiences new researchers face. I applied for a Ball State University internal grant through Sponsored Projects Administration office for funding to continue research in T-cell acute lymphoblastic leukemia. In this project, I analyze the process I went through, why I chose to write what I did, and what I would choose to do differently. My hope is that by explaining this process from an undergraduate perspective, others can learn from my challenges and experiences.

PROCESS ANALYSIS STATEMENT

In scientific research, as in many other areas of academia, it is imperative to receive grant funding in order to carry out the study to reach an end goal. As I am going into the research field, I thought it would be beneficial to study how to properly write a grant to aid in the research I am currently performing. The first step was finding the right grant program; for me, it was the Ball State University's Sponsored Projects Administration internal grant. This gave me experience in two distinct areas: first, in general grant writing, and secondly, in tailoring the grant to an audience who had little to no science background. This caused me to analyze my work further and determine how to write what I was studying in a language the non-scientist could understand. This was a challenge, as I am accustomed to writing in a manner where scientists understand the complex terminology commonly used in research papers and presentations. Because of this, I had to complete numerous rewrites of the final draft. After advice from my mentors, I was able to complete the grant proposal and received funding for my research project. I will now share with you, the reader, my overall experience.

THE GRANT APPLICATION PROCESS

In order to identify which grant program is most relevant for your work, it is crucial to look into the types of grants available, the application requirements, and the audience who will be reviewing the proposed work. There are many types of external grants available from local, state, federal, private, and public agencies, as well as internal and organization-based grants. I chose an internal grant from the Ball State University (BSU) Sponsored Projects Administration, which gives students experience in grant writing without necessarily completing preliminary research. During the 2015-2016 fiscal year, the BSU Sponsored Projects Administration received 27 undergraduate applications and 135 graduate applications, funding 19 and 101 grant requests, respectively. In total, $3,950 was awarded to fund 70% of the undergraduate applicants and $269,819 was awarded to fund 75% of the graduate applications (Sponsored Projects Administration, 32). Other grants available to student researchers include the Ball State Chapter of Sigma Xi (local), the Indiana Academy of Science Senior Research Grant (state), or the Sigma Xi Grant-In-Aid Grant (national), and the Federal Pell Grant, which needs to be applied for in conjunction with the primary investigator. However, the Federal Pell Grant is usually applied for as either a scientist who is a post-doc or lead researcher or as a secondary author on the grant. Most of these will require accompanying recommendation letters from your research mentor or others familiar with your work and leadership skills (more information on this in the next article "How to write a good recommendation letter," this issue of Fine Focus).

The requirements for the BSU internal application included a cover sheet, a budget, a project design, references, letter of support, and a curriculum vita. The cover letter is designed to give a brief summary of the research and gives readers a quick glance as to what the rest of the documents are about (Appendix I). Attached to the cover letter was the budget, which explained what the money would be spent on (Appendix I). It is common for granting organizations to have stipulations on how much can be funded. The BSU internal grant program limited undergraduate applicants to $300 or less and graduate applicants to $500 or less; it is important not to request more than the maximum allowable in the grant description. Other grants, such as the Indiana Academy of Science Grant, allows up to $2500 to be requested by applicants. Although more uncommon, another potential decision factor for the amount requested includes matching policies, where a granting agency will match that raised from additional resources, with these latter sources usually stipulating their contributions and support in an ancillary letter that will comprise part of the overall grant application package.

There are five general categories for the BSU internal grant program: contractual, supplies, travel, participant costs, and other. Contractual grants fund software licenses, such as needed statistical software to analyze data (or similar). Supplies include any reagents, materials, or small benchtop equipment needed by the researcher. In this case, the BSU program would fund $300, while the remaining project costs would be funded by additional sources, such as the Biology Department and the Honors College. This is typical for most primarily undergraduate institutions (PUIs). Travel costs include travel to research sites or participation in research conferences. Participant costs, such as conference registration or abstract submission costs, go hand-in-hand with travel costs. Other costs include everything not described above, such as purchasing larger pieces of equipment. My advice would be to discuss the budget categories, needs, and spending timeline with your research mentor before drafting the budget, then carefully edit it together, including sources for each line item, quantity, and catalogue numbers.

Following the development of technical budget comes the budget narrative, where the applicant affirms why all of the costs described in the budget are necessary. In the budget narrative, it is important to describe why something has to be purchased, particularly items that seem conspicuously expensive, such as an antibody, enzyme, or unique reagent available from only a single source. These reasons should be clearly stipulated, and shipping costs (ex. dry ice shipping, hazardous material costs, and related) should be included and mentioned in the budget justification as well.

It is important to note that some research grants have caveats, such as the funding cannot be spent on compensation. This means the funding received could not be paid to myself or any other lab assistant

to compensate for work completed. Larger grants, such as those awarded by the National Institute of Health and National Science Foundation, have some additional funding categories, such as compensation and university costs. Smaller grants usually will not pay for compensation because of the additional resources that are required to pay employees, and would require tax forms, benefits, or other logistics usually out of the realm of smaller internal grant opportunities. Larger foundations have this resource and can go through the additional work to provide this benefit to researchers.

Another point worth mentioing would be indirect costs that go back to the applicant's university. At BSU, this can go as high as 23% of the grant awarded, some of which will go back to the Sponsored Projects Administration to fund student and faculty research, or to other administrative units. Other universities have a much higher indirect cost, which may deter funding organizations from approving and awarding a grant. However, most or all internal funding opportunities for undergraduate projects will not involve any discussion of indirect costs being factored into the budget.

When it comes to who applies for the grant, there is a distinct difference between the primary investigators and co-investigators. The primary investigator is the one who comes up with the initial idea for the research and applies for the larger grants. Typically, granting organizations want to hear from the person who had the idea for the overall project when granting large sums of money. However, for the BSU grant, the title of principal investigator was switched to the applicant and not the person who came up with the idea. In this instance, the student was the principal investigator instead of the professor. To decide what the idea for the project would be, my mentor, Dr. James Olesen (BSU Biology) and I looked at previous work I had completed and decided to create a project that was a continuation of it.

The third section of a grant is the project design, which has many distinct parts: an overview, background, goals and significance, and the research method timeline, each approximately a paragraph in length (Appendix II). The overview of the project is similar to the introduction of an essay, in that it explains what will be attempted in the proposal and why the research is significant. Under background information, the information about the history of the project and the current status is explained. The writer lays out what is being studied in a way grant reviewers will understand. This is where it becomes imperative that one uses appropriate terminology. In terms of the BSU internal grant proposal, the audience included a panel of faculty from many different fields, such as history, math, english, and the sciences. Not all grants have a wide range of reviewers, as most are discipline specific. When they are discipline specific, grants can be more detailed as the readers will be more knowledgeable as to what the project is designed to accomplish, what the grant will be spent on, and why the project is important to society at large. Since the BSU grant proposal was open to a wide range of undergraduate majors, reviewers came from many different departments across campus to ensure a fair review of all proposals. Thus, the writing had to be simplified to a level where most people reading could understand. If technical terminology had to be used, a glossary of terms was added to help without taking away the professionalism of the application (Appendix III). Overall, this section should contain some level of detail, including any preliminary data, as it explains the concepts behind what is being studied or proposed.

Finally, appropriate references are included and formatted appropriately to the field in which the grant is being written (Appendix IV).

The third section of the project design includes explaining the overall goals or specific objectives of the research as well as the significance of the research. In research proposals, there may be two or three major goals that are presented. In this section, the applicant explains those goals to the reader and describes what they will do to attain them. In my lab, the ultimate end goal is to determine how the transcription factor TAL-1 impacts leukemic T-cells and prevents cell death. The specific objectives are what the independent researcher is attempting to accomplish, such as what each lab tech is trying to accomplish to help reach the main goals. In Dr. Olesen's lab at Ball State, there are three other students all working on different projects, so my specific objectives are different from theirs. My goal is to understand if TAL-1 influences caspase-10, thus preventing cell death. The significance of research is being able to take the knowledge outside of the laboratory and apply it to the real world. For this specific project, the significance is to be able to explain why the leukemic T-cells do not undergo death when treated with a chemotherapeutic drug and then use what we learn to help develop therapeutic treatments that may be able to treat T-cell Acute Lymphoblastic Leukemia.

The final section of the project design includes the research methods and timeline of the project. The methods section includes a description of what experiments and protocols will be utilized and are written in a way that others could follow. When detailing the protocols, not every step is included for sake of brevity, but other grants require that level of detail. Typically, the larger the grant, the more detail the granting agency will require. This description of the methods shows reviewers the applicant has thought through the project, including what protocols will be best to run, and any additional experiments if the project does not go as planned (contingency plan). The timeline gives the granting agency an expected deadline as to when the project will be completed. This gives the reviewer a sense of how long the research will take and whether the timeline is feasible.

After the project design is completed, at least one letter of recommendation is needed. In the case of the BSU internal grant, a letter of recommendation was required from the faculty mentor of the student who submitted the application (Appendix V). The letter of support must include the *via*bility of the project, a description of how the project will contribute to society, how the project fits with the aspirations of the student, and what role the student plays in the research of the faculty member. This letter of recommendation gives credibility to the student or applicant as to why the funding is needed and verifies the importance of the project.

Finally, a curriculum vita (CV) is required as documentation of the applicant's abilities; the CV is generally required for all grant programs, regardless of the funding level or source (Appendix VI). A CV is required to provide support as to the applicant's background and to chronicle other work they have done in the chosen field of research. For the BSU grant, the CV included education, research experience, and awards and honors sections. Under education, the university attended, majors, grade point average, and expected date of graduation is listed. Additionally, examples of relevant coursework that have contributed to the skills or experience of the student may

be included in this section. The research experience section is where all of the applicant's previous professional research is explained. The professional title, lab, location, and dates are listed for each professional lab in which the applicant has worked. This also includes a brief description of the research completed and the job responsibilities. Responsibilities can range from skills, such as running specific experiments, to training new lab assistants. Furthermore, attendance at conferences should be listed. By attending conferences, either local, regional, or national, this shows the applicant is capable of explaining his or her research to a group and is invested in the dissemination of the research. For some of the conferences, a peer-review of the applicant is done in order to determine if a presentation should be accepted. For example, our annual Ball State University Student Symposium only accepted 100 presentations out of many more applications. By being accepted into a conference, the applicant has shown the significance of the work they are doing and the ability to communicate that significance to others. Finally, many granting organizations want to see if the investigators have been publishing their work and how frequently prior to granting larger sums.

Finally, the last component of a grant is a final report that is submitted to the granting organization. After a designated amount of time, perhaps 12 months following the award, the recipient of the grant is required to submit a report describing how the project went, what was discovered, and where the project will go in the future. For the BSU internal grant, this gives the administration an opportunity to make sure the research was completed, while also validating the faculty mentor is worth funding in the future, should other students apply for a grant from his or her lab.

If funding is denied, the hope is that the review panel would provide a ranking and/or a thorough overview of reasons on what aspects of the proposal were lacking or poorly written, in order to improve for a resubmission during the next cycle. Occasionally, different reviewers may generate comments that contradict one another, or that seem out of place. However, a good review process will minimize the frequency of superficial or incorrect criticisms, which should be resolved during the panel's deliberation and ranking discussion, in order to allow the submitter to learn how to make the proposal stronger and more clearly written after revisions. Your research mentor will assist you with this phase as well, and offer encouragement along the way so that you are not hopelessly frustrated.

Overall, this exercise has helped me become a better researcher by providing me with experience in how to fund a research project. I now know how hard scientists have to work to come up with hypotheses and perform research. They have to continuously publish their work and make sure the significance of the work is known. However, most people do not realize the importance of grant funding and how it is the foundation of any research. By starting grant writing in my undergraduate years, I have been able to learn how to apply for grants, what is required, and why it is important. With this understanding of the process of applying for grants, I hope to be able to better fund the research of my future employers based on the knowledge I gained from writing the BSU grant.

Overall, grant writing is an art form that helps progress all areas of research, whether it is in the arts or in the sciences. This project has shown the importance of reworking different pieces of writing to cater to all those who may read the application and all the necessities that go into grant writing. It is not an easy feat for one to obtain funding for their research and should be a celebrated moment in the applicant's life when he or she finally does receive funding.

WORKS CITED

Sponsored Projects Administration. (2016, November 17). Ball State Research 2016. https://issuu.com/ballstatespo/docs/research_2016_1116

APPENDIX I

BALL STATE UNIVERSITY. *Sponsored Projects Administration*

ASPiRE Internal Grants

Student Application Coversheet

Submission Date: Fall | Competition: Undergraduate Research | Project Dates: January 1 - December 31

Name (Project Director)	Department	Preferred E-Mail	Position
Amanda Baskfield	Biology	andingman@bsu.edu	Undergraduate
Name (Project Co-Director)	Department	Preferred E-Mail	Position
Faculty Mentor (Required)	Department	Preferred E-Mail	Position
Dr. James Olesen	Biology	jolesen@bsu.edu	Tenure

Project Title: Assessment of Caspase-10 Expression Levels in T-Cell Acute Lymphoblastic Leukemia

Summary: (Two to three sentences single spaced)

Caspase-10 is a protein found in the cytoplasm of a cell that is responsible for initiating programmed cell death, also known as apoptosis. In this study, we assess how expression levels of caspase-10 change with the expression levels of TAL-1, a transcription factor that is thought to prevent apoptosis from occurring in malignant T-cells.

Is this proposal a resubmission of a previous application? ○ Yes ◉ No

If yes, what was the original competition? _____ Date of competition _____

Compliance

Compliance issues? No compliance issues with this project

Compliance Status: Not Applicable

Budget

	ASPiRE Request	College/Dept Support	Other Support
Contractual (incld. software licenses)			
Supplies, Materials, Minor Equipment	$300.00	$134.00	
Travel			
Participant Costs			
Other			
Totals	$300.00	$134.00	

Budget Narrative: Please explain & detail all costs requested in the table above in the space provided below.

The cost to purchase the inactive form of Caspase-10 is $434 from Abcam, a company that sells high-quality antibodies that aid in the analysis of protein expression in various cell types. The $300 provided by the Aspire grant will be used to purchase the antibody, with the remaining $134 being paid from Dr. Olesen's research fund in the Department of Biology. All other supplies and reagents needed to carry out the planned experiments are in place. By being able to understand how this protein is being regulated, we will be able to better understand the characteristics of T-ALL and why natural cell death is not occurring.

APPENDIX II

EXECUTIVE SUMMARY:

T-cell acute lymphoblastic leukemia (T-ALL) accounts for 15-25% of all acute lymphoblastic cases in children and adults. Characterized by a resistance to chemotherapy, this cancer originates from white blood cells (T-lymphocytes) in the bone marrow. After becoming malignant, these cells continue to over-proliferate in the blood stream and upset the balance of the immune system. The development of new treatments for T-ALL has been stalled due to the complexity of the molecular signaling pathways involved (1). T-ALL is thought to occur from the ectopic expression of a transcription factor, known as TAL-1, which has the ability to bind to DNA and influence the expression of additional genes (2). It is thought that TAL-1 may impact the expression of genes, especially those influencing the apoptotic or death cascade, thus allowing a cell to avoid death induced by chemotherapeutic treatments. Thus, the influence of TAL-1 needs to be further characterized, which is what this study is meant to accomplish.

Background Information:

Further examination of important proteins that are potentially targeted by TAL-1 is necessary. If the apoptotic-signaling pathway in a malignant T-cell is understood, this could serve as vital information for the development of a treatment therapy for T-ALL. In normal cells, the apoptotic pathway is key in removing unwanted or abnormal cells through a well-defined series of events. One protein that has shown to be critically important in the apoptotic cascade, which may be influenced by TAL-1, is caspase-10. Found in the cytoplasm of the cell (outside the nucleus), caspase-10 acts as an initiator of the apoptotic pathway. If activated, this protein can go on to activate other caspases responsible for the destruction of the cell (3). Thus, caspase-10 has the ability to activate apoptosis in the cell. By determining expression changes in caspase-10, the characteristics of the apoptotic signaling pathway involved in T-ALL can be further determined. These insights are crucial to the development of better, more targeted drug treatments for this rapidly spreading cancer.

GOALS, OBJECTIVES, AND SIGNIFICANCE:

Previous research from our lab suggests that TAL-1 may negatively influence the induction of apoptosis, but further investigation is needed to understand the exact mechanism of this inhibition. The goal of this research proposal is to determine if TAL-1 influences the expression of an important initiator caspase known as caspase-10, thus promoting survival after etoposide drug treatment. The Jurkat T-cell line will be used to mimic T-ALL and inhibition of apoptosis will be assessed through examination of protein expression levels. In the end, a better understanding of the proteins and molecular interactions influenced by TAL-1 may be realized.

Research Methods and Timeline:

The Jurkat T-cell line will be cultured in an RPMI/10% bovine growth serum media and maintained at 37O C and 5% CO2 to promote cell growth and division. Drug treatments with etoposide will be performed over a 24 hour period at concentrations of 0 μM (control), 1 μM, and 5 μM and whole cell lysate protein extracts will be created from all three cell populations. A Bradford Assay will determine the protein concentration of each extract. The extent of apoptotic induction will then be assessed using Western blot analysis, immunofluorescence, and flow cytometry. For Western blot analysis, polyacrylamide gels will be loaded with the extracts and proteins will be separated by electrophoresis. The separated proteins will be transferred to a nitrocellulose membrane using a semi-dry transfer apparatus. After transfer, the membrane will be exposed to a primary antibody against caspase-10. Expression levels of caspase-10 will be analyzed and quantified using a Li-Cor imager system. Furthermore, immunofluorescence will be performed where cells will be fixed with paraformaldehyde, air-dried onto slides, and incubated with the caspase-10 primary antibody. Next, cells will be incubated with a secondary antibody containing a fluorescent tag, which will bind to the primary antibody. A Zeiss fluorescence microscope, fitted with a UV light source, will be used to excite the fluorochrome, allowing for the visualization of caspase-10. Finally, flow cytometry will be used where cells will be washed in PBS and then exposed to permeabilization buffer to disrupt the plasma membrane so the caspase-10 antibody will enter cells. A secondary antibody containing a fluorochrome tag will be added and will join to the primary antibody so caspase-10 can be visualized in cells. This research project will be completed during Spring Semester of 2017 and presented at the upcoming Ball State Student Symposium and also at the 132nd Indiana Academy of Science meeting in March 2017.

APPENDIX III

GLOSSARY OF TERMS:

Apoptosis: the highly ordered and timely process of programmed cell death, which can be beneficial to an organism in the removal of unwanted or damaged cells.

Caspase-10: a protein that activates/cleaves other proteins (protease) in the apoptotic cascade.

Concentration: the amount of a protein, in μg, that is present in 1 μl of whole cell lysate

Electrophoresis: a technique used to separate proteins through migration in an electronegative field

Ectopic: the expression of a biological molecule in an abnormal location in an organism or its cells.

Etoposide: a chemotherapeutic drug that stops cell growth and division, while also moving the cell into apoptosis.

Flow Cytometry: a laser-based technology used in cell counting, sorting, and the detection

of proteins.

Immunofluorescence: a technique used to determine the presence of a protein or antigen biomarker inside of a cell through antibody binding.

T-ALL: T-cell acute lymphoblastic leukemia is a rapidly spreading malignant cancer of the blood cells and bone marrow.

TAL-1: a transcription factor involved in blood cell development. Abnormal expression may result in various cancers such as T-ALL.

Transcription Factor: a protein that binds to DNA sequences and controls the expression of other genes.

Western Blot Analysis (protein immunoblot): an analytical technique that can detect the presence of specific proteins isolated from cells.

Whole Cell Lysates: protein extracts created from cells, which can be used as samples in Western blot analysis.

APPENDIX IV

RESOURCES:

1) Chiaretti S. Foá R. T-cell acute lumphoblastic leukemia. Haematologica. 2009;94(2):160-162. doi:0.3324/heamatol.2008.004150.

2) Soumyadeep, D., Curtis, D., Jane, S., & Brandy, S. (2010, May). American Society for Microbiology. Retrieved April 06, 2016.

3) Overview of Apoptosis (2016). Cell Signaling Technology, Inc.

APPENDIX V

BALL STATE UNIVERSITY

College of Sciences and Humanities
Department of Biology

Muncie, Indiana 47306-0440
Phone: 765-285-8820

Chairperson
Aspire Undergraduate Student Grant
Ball State University

November 4, 2016

To Chairperson of Review Committee,

I am writing this letter of recommendation in support of Amanda Baskfield, who is applying for an Aspire Undergraduate Student Grant. I have known Amanda for approximately two years and first met her when she approached me about research opportunities in my lab. She started in my lab during Spring Semester 2015 and has been exposed to many experimental techniques and has been a part of several ongoing projects. The proposed project represents an important part of her overall research plan that she needs to complete. This project will also give Amanda additional research experience that should prove useful as she builds on her skills to help in future endeavors. Over the last year, she has completed preliminary research that will be presented at the Sigma Xi Undergraduate Research Conference in November 2016. This is commendable for an undergraduate student.

Amanda's project is based on previous research from my lab that indicated Jurkat cells, which ectopically express Tal-1 (a transcription factor), do resist apoptotic induction when treated by the chemotherapeutic drug called etoposide. She now needs to assess how this apoptotic resistance is mediated and how Tal-1 is potentially involved. There is not much information in the primary literature suggesting how Tal-1 negatively impacts cell death, thus it is important to perform experiments such as what she is proposing. We need to continue to look for the molecular targets of this transcription factor to better understand how gene expression patterns change in T-cell acute lymphoblastic leukemia. With an increase in our understanding, this can lead to more insights into how Tal-1 contributes to the over-proliferation of cells and the resistance to death. Overall, the viability of the project is great and any information gained could prove useful in future cancer therapies.

The proposed research that Amanda will complete represents an area of ongoing research in my lab. My last several students laid the groundwork for what she is working on now and I envision this work to continue for the foreseeable future. Thus, it is crucial that this project be completed to give us a better idea of what is happening at the cellular level. She is asking for support to purchase a caspase-10 antibody to be used for Western blotting, immunofluorescence and flow cytometry experiments. By purchasing this antibody, she can assess an important initiator caspase that triggers the apoptotic cascade. Since Jurkat cells resist apoptotic induction, there may be a change in the expression level of this protein due to the presence of Tal-1. Other miscellaneous reagents/supplies will be provided to Amanda through research support provided to me by the Department of Biology. Support through this grant will afford her, and in turn, my lab, the ability to assess if/how Tal-1 affects important proteins involved in the control of apoptosis.

I also want to mention that another undergraduate working with me, Mallori Wisuri, is submitting an Aspire Grant at the same time. While my letter and the overall grant proposals are similar, they truly are different, albeit, related projects. Both of these students are looking for potential target proteins whose expression might be changed in Jurkat cells treated with etoposide. Both projects are equally important and significant in their own right.

In conclusion, I feel Amanda can successfully complete this portion of her research and it will provide key data to be used by my lab in future projects. I hope your committee will look favorably on her proposal and if you need additional information or clarification, please feel free to contact me.

Sincerely,

James B. Olesen

Dr. James B. Olesen
Department of Biology
Ball State University
Muncie, IN 47306
(765) 285-3510
jolesen@bsu.edu

APPENDIX VI

Amanda N. Baskfield
3015 North Oakwood Avenue, Apt 439.
Muncie, IN 47304
Phone: 317.478.0841
Email: andingman@bsu.edu

Education

Ball State University, Muncie, IN
Majors: Biology and Finance
Grade Point Average: 3.67
Graduation (Expected): May 2017

Selected Examples of Coursework: Molecular Biology, Cancer Biology, Undergraduate Research, Cell Biology, Microbiology, and Genetics

Research Experience

Research Assistant
Dr. James Olesen, Muncie, IN January 2014 – Present

- Run protocols, such as Western Blot Analysis, Immunofluorescence, and Flow Cytometry, in order to demonstrate protein expression in T-cell Acute Lymphoblastic Leukemia.
- Alter protocols to fit within the dimensions of the study, in order to obtain the best possible results.
- Train new lab assistants in the protocols and the expectations of the lab, while reporting to Dr. Olesen about their skills.
- Determine proper techniques to fit within the yearly budget of the lab.
- Present findings at local and national conferences, including National Collegiate Honors Council, Sigma Xi Annual Meeting, Indiana Academy of Science Annual Meeting, and Ball State Research Symposium.

Awards and Honors

Honors College Undergraduate Fellowship, Sigma Xi Scientific Research Society Member, Dean's List, Honors College, Golden Key Society Member, Society for Collegiate Leadership and Achievement, Miller College of Business Honors Program, Receiver the Scholarship from the *Estate of Wally Miller*, Flanner and Buchanan Volunteer of the Month, and Circle K International Board Member of the Month for the State of Indiana

HOW TO WRITE A GOOD RECOMMENDATION LETTER

BARBARA STEDMAN, Ph.D.
DIRECTOR OF NATIONAL AND INTERNATIONAL SCHOLARSHIPS
BALL STATE UNIVERSITY

In my work as the Director of National and International Scholarships at Ball State University, I see dozens of letters of recommendation each year, the vast majority of them written by faculty. Over the past decade I've learned that some faculty members may view writing a letter of recommendation as a burden, as an interruption to their own research agendas or other activities they consider more important than supporting a student's application. These recommenders may dash off a letter in 15 minutes, using the same trusty boilerplate that they've used for a hundred other students.

Thankfully, though, I've learned that most of our faculty view letter-writing as a privilege, as an important way to help advance a student's academic and professional careers. These letter-writers easily spend an hour or more crafting and editing a letter, and these are typically the instructors who are asked again and again to provide letters for students' applications for scholarships, internships, and graduate school.

From the students' perspective, asking for letters of recommendation may seem like a crapshoot: letters are the one component of an application that isn't under their control, and it typically remains unseen, the content unknown. When the competition for a national scholarship is especially fierce, however, a strong letter can be the factor that ultimately determines a student's chances of success.

In the following pages I offer some general advice for writing strong, persuasive letters of recommendation for scholarship applications in general, along with some specific advice for STEM-based scholarship programs, particularly the Barry M. Goldwater Scholarship and NSF Graduate Research Fellowship (NSF GRF)[1], two of the most important and visible scholarships available to STEM students in the U.S.

1) TAILOR EACH LETTER ACCORDING TO THE SPECIFIC CRITERIA OF A GIVEN SCHOLARSHIP.

Resist the urge to rely on your go-to boilerplate when writing a letter for a major scholarship competition, and don't simply change the name of the scholarship when recommending the same student for more than one competition. The applicant should provide you with a list of the criteria for a given scholarship competition; if that hasn't happened, ask the student for the criteria or go to the scholarship program's website, where you may find overt guidelines and advice for letter-writers.

For STEM-based scholarships, research experience and future potential in research are often the most important criteria. Goldwater Scholarship applicants, for example, are explicitly advised to solicit letter-writers who can discuss their "ability, interest, and experience in conducting research." Recommenders should therefore focus much of their attention on past research experiences, whether in the lab or the field, and should also comment on an applicant's relevant personal traits: "the motivation, creative thought, stamina, and ability to collaborate that are characteristics of those successfully practicing" in STEM research.

For the NSF Graduate Research Fellowship, research experience and potential – including the feasibility and significance of the applicant's Graduate Research Plan Statement (one of two essays submitted with the application) – should again be focal points in a letter of recommendation. NSF GRF letter-writers are also asked to "include comments on the applicant's potential for contributing to a globally-engaged United States science and engineering workforce" and to take special care to address the NSF merit review criteria – i.e., the Intellectual Merit and Broader Impacts criteria.

In addition to providing information about the scholarship criteria, applicants should have provided you with reminders of their past accomplishments and perhaps even a request to focus on a particular experience (e.g., "I'd appreciate it if you could discuss my leadership of the Human Genome Project"). They may even want you to discuss an experience or quality that they haven't had opportunity to elaborate on in their part of the application. And while it's always a good idea for recommenders to read essays that applicants have written for a scholarship application, doing so is especially important for the NSF GRF, as noted above.

2) DON'T SIMPLY SUMMARIZE THE APPLICANT'S ACTIVITIES AND ACCOMPLISHMENTS; INTERPRET AND EVALUATE THEM.

Many letter-writers believe that eloquently paraphrasing a student's résumé is useful and persuasive. It isn't. Students already will have provided the content of their résumés in the application, so restating that same information in your letter provides no new information for the reviewers.

Instead, bring your knowledge and experience to bear upon your evaluation of this student, to help the readers understand what the applicant's activities and accomplishments mean, perhaps in the context of other outstanding students. How does this student stand out from the crowd of other brilliant, visionary, accomplished students you've known and worked with? Sometimes numbers are useful in making that case. For example, stating that a student is in the top 1% of all undergraduates you've taught over a 30-year teaching career conveys a powerful message. Or favorably comparing the applicant to a previous student who went on to an illustrious research career can be useful, too.

Joe Schall, author of Writing Letters of Recommendation, states that good letter-writers "invite us to imagine ourselves in the presence of the student" and help us to "know the candidate well, in fact to admire the student." In creating this "invitation," consider what kind of impressions the student has made on other faculty in your department or on other students; you may want to quote one or two of them.

Even while inviting readers to admire the student, however, avoid grandiose hyperbole. Some scholarship review committees don't even like to read the word "perfect" in a letter of recommendation (i.e., "Jane Smith is a perfect candidate for the ___ Scholarship"). Readers don't expect any candidate to be perfect and without flaws – which raises the question of addressing failures and weaknesses in a letter of recommendation. Should you include them? If the student has significant weaknesses and unaddressed failures, you may first need to reconsider whether you feel comfortable providing a letter for him or her.

In general, however, you should discuss failures if they have led to positive growth or if they help explain a gap or weakness in a student's record. The Goldwater Foundation even offers this specific advice to recommenders: "If there are any special circumstances or challenges the student has had to face that have negatively affected the student's performance or that the student has faced and has successfully addressed and performed well in spite of these challenges, please discuss these in your letter." I would simply add the caveat that if such challenges are personal in nature, ask the student if you may mention them in your letter.

3) SUPPORT YOUR POINTS WITH SPECIFIC, ILLUSTRATIVE DETAILS AND ONE OR MORE

1. The Barry M. Goldwater Scholarship is aimed at identifying and supporting sophomores and juniors who will become the next generation of our nation's top STEM researchers. The NSF Graduate Research Fellowship provides three years of generous support to outstanding graduate students (including those who will enter their first year of graduate school) in a wide range of the sciences, including social sciences. Both programs are federally sponsored and funded.

PERSONAL ANECDOTES.

Don't leave your readers asking "How?" or "In what way?" If you say a student has tremendous initiative, describe an incident in which he or she demonstrated that initiative in a meaningful way. Flesh out evaluative statements – "Jane uses both her senses and her intuition well in the lab" – by offering an anecdote in which you saw Jane's senses and intuition at work. Help the readers experience that scene through engaging description; convey the surprise and pleasure you experienced when you first saw her talents in action.

You should also show readers that you know this student as more than simply a good test-taker or lab worker. Identify noteworthy character traits, adopt a warm and personal tone in your writing – there's no need to refer to the student as "Miss" or "Mr." – and be sure to employ memorable details. After the scholarship review committee members have finished reading several dozen application packets, what will they remember from your letter? The more that your voice comes through, the more compelling your letter will be.

4) CONSIDER THE STRUCTURE, LENGTH, AND OTHER FORMATTING DETAILS.

Unless you are instructed otherwise, I recommend the following general guidelines.

a) Greeting: Address the foundation or committee, rather than "Dear Sirs," or "To whom it may concern."

b) Opening paragraph:

• State what you're recommending student for.

• Indicate the length of time and circumstances in which you've known the student.

• If needed, provide a brief statement of your qualifications for evaluating the applicant, but don't make the letter about you; the review committee will not care how accomplished you are.

c) Readability:

• Don't go smaller – or larger – than 11 or 12 point Times New Roman, 10 point Arial, 11 point Calibri, or a comparable font and size.

• Single-space your letter, with adequate paragraph breaks.

• Don't use visual gimmicks. It's fine to boldface or italicize a few key words, but don't overuse either formatting tool, and don't use "splashy" fonts, in an attempt to draw attention. I once had a faculty member put a student's full name in 14-pt. Broadway font throughout his letter, thinking that he would help readers remember the student's name. Yes, the reviewers might have remembered the student's name, but not for the right reasons!

d) Length: For most scholarships, aim for 1½ to 2 pages or about 1,000 words. A one-page letter may suggest you have little to say, and three pages may try the patience of committee members who are reading and evaluating dozens of applications.

Be sure to read the instructions for each specific scholarship program, though, as you may be given a character, word, or page limit. Recommenders for the NSF GRF are limited to two pages, and those for Goldwater Scholarship are allowed up to three pages. Other formatting guidelines are also specified by each program, too, including font options and the width of margins.

e) Submission protocol: Because the vast majority of scholarship applications are now online, you'll likely be required to copy and paste your letter into a text box or to upload it as a Word or PDF file. When copying and pasting into a text box, you don't need to worry about letterhead or signature. When uploading a file, though, you'll usually need to have both letterhead and your signature.

For uploaded letters, you may be given the option of submitting a Word document, but I recommend going with a PDF instead. If you want to print out your letter and then scan it as a PDF, be sure to set your scanner resolution to 300 dpi or higher; 200 dpi will make the text grainy and potentially annoying to your readers. If you have your letterhead and signature saved as a .gif or .jpg (or other image file), however, you may find it easier to simply insert the letterhead and signature into the Word version of your letter and then "Save As" a PDF.

f) Proofread: Spell the student's name and the scholarship's name correctly. I've seen students called the wrong name in letters, have their names spelled two different ways, identified by the wrong pronoun, and recommended for the wrong scholarship!

For online submission of letters, too, be sure to preview the letter before hitting "submit," to check for formatting problems created by copying and pasting from Word into a text box.

5) TRY NEW STRATEGIES FOR IMPROVING YOUR LETTERS OF RECOMMENDATION.

If you're not happy with the letters you've been writing, or if you've often found yourself at a loss to write a substantive letter for some applicants, these strategies might help:

a) Meet with the student in person, to discuss the scholarship and the student's accomplishments and future plans. Even if the student sends you detailed written information, a 20-minute conversation may help you come up with convincing anecdotes or help clarify what you want to focus on in your letter.

b) Think of a letter of recommendation as a rhetorical challenge: You're building an argument about a student's intelligence, insight, ambition, and potential to have a significant impact on his or her field of study, profession, and perhaps even society. You're also building a persuasive argument that this student is a good fit for this scholarship. What do you need to tell the readers to convince them of your broad statements about the student?

c) If you simply feel "stuck" and can't figure out how to write more than half a page about a student, try this strategy: Identity three of the student's qualities that make him or her especially well qualified for the scholarship or otherwise stand out from the crowd. Write a paragraph or two about each quality, providing supporting evidence and at least one anecdote to illustrate each point. While the strategy itself may sound formulaic, the results are usually distinctive and convincing, allowing the readers to get a clear picture of that particular applicant.

6) BE ETHICAL.

Never ask a student to draft his or her own letter of recommendation. But do ask the student to give you written information about the scholarship, plus reminders about past papers and projects and other relevant accomplishments that you're especially well-qualified to discuss.

If you can't write the kind of letter that will help a student be a strong contender for a scholarship – even after trying some of the strategies provided here – please say so from the start. Writing an unusually short or unenthusiastic letter only hurts the student.

Even though your letter is almost always considered confidential by scholarship foundations (you'd be alerted to any rare cases in which that isn't the case), and certainly letters for the Goldwater Scholarship and NSF GRF are kept confidential , you're welcome to share your letter with an applicant if you like. In fact, you may want to fact-check the content of your letter or simply let an applicant know how highly he or she is regarded. However, you should never comply with a student who asks you to hand over your letter without first confirming the scholarship application's protocol.

Writing a strong letter of recommendation takes time and careful thought, but for scholarship applicants your letter can be the deciding factor for a review committee debating between one or more equally qualified candidates. And even for those students who may not win a scholarship, your letter will likely provide a useful foundation for future applications, whether for other scholarships, internships, or graduate school.

The investment of time and energy is worth it, so throw out that boilerplate when recommendations really matter. Students will be grateful, and you may help provide them with an opportunity that will open door after door in their academic careers and professional futures in science.

BOOK REVIEW: LAB MANUAL – "MICROBIOLOGY– THE LABORATORY EXPERIENCE" (STEVE KEATING)

W.W. NORTON & COMPANY, ISBN 978-0-393-92364-3

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INTRODUCTION

In Microbiology: The Laboratory Experience, author Steve Keating takes lab manuals where they have not gone before. He states his purpose, "... to write a microbiology lab manual as a means of speaking directly to students to increase their interest and understanding of the subject and to decrease the number of mistakes." Through his dedication to providing students with a manual that is interesting, simple yet informative, engaging, and inclusive, Keating presents the most impressive lab manual I have seen as an undergraduate student.

CONTEXT

The bulk of the manual is made up of 7 Units: (1) The Fundamentals, (2) Staining Microbes, (3) Effects of the Environment on Microbial Growth, (4) Identifying Medically Significant Bacteria, (5) Applied Food and Water Microbiology, (6) Microbial Ecology, and (7) Viruses; each of these sections an umbrella for the various other important subsections. Each section provides a significant amount of relevant background information for the corresponding laboratory experiment. Unit 1: The Fundamentals is accurately titled as it covers laboratory procedures which will be utilized in each of the subsequent units (the microscope, working with and storing pure cultures, quantitative plate counts). Unit 2: Staining Microbes covers each of the familiar microbe staining topics including general staining and cell morphology, the gram stain, acid-fast stain, and endospore stain. However, this manual goes so far as to take the idea of the normal one-day staining lab and separates it into individual staining labs, making the information for each more detailed and easily digestible. Unit 3: Effects of the Environment on Microbial Growth is one of the more expansive units (along with Unit 4). The unit covers environmental factors including: media components, bacterial enzymes, temperature, pressure, pH, oxygen, UV, disinfectants, antiseptics, and antibiotic sensitivity. It also provides techniques for calculating the minimum inhibitory concentration (MIC). Unit 4: Identifying Medically Significant Bacteria includes techniques for isolation, PCR, and identification of both known microbes (*streptococcus* spp., *enterococcus* spp., *staphylococcus* spp., *micrococcus* spp.) and unknown bacteria. It also provides elaborate detail on the *Enterobacteriaceae* family with corresponding identification techniques. Units 5, 6, and 7 though short, continue to do an adequate job of providing contextual information that is thorough and accompanying it with procedures that give students real-world laboratory experience. Unit 5: Applied Food and Water Microbiology deals with milk microbiology, water, meat, and wine microbe analysis. Unit 6: Microbial Ecology cover topics such as the Nitrogen cycle, Winogradsky columns (something that I have never encountered in my microbiology experience as an undergrad student), and antibiotic producers in soil. The final unit, Viruses, focuses primarily on understanding bacteriophages and how to type them.

Aside from providing sufficient background content and real-world lab experience, Keating begins each laboratory procedure with clearly outlined learning objectives and ends the lab with conceptual post-lab questions. Learning objectives allow students to get an idea of the key concepts and techniques they will be learning about/performing, while the post-lab questions facilitate conceptual understanding and real-world application of each procedure. What separates this manual from others I've come across is that Keating refuses to cut corners. He makes sure that the manual is an object which can stand on its own, providing sufficient content in all areas, no matter where your instructor may lack.

LANGUAGE

It is obvious that Keating has students in mind when noting the overall clarity of the lab manual. As a 4-year science major, I still find myself getting lost in some scientific jargon. However, with this manual, I did not once experience that lost feeling. I could better understand the information and techniques presented, and I was able to reflect on them through the post-lab questions, without feeling as though the questions were set out to confuse me.

FIGURES

One of my favorite things about Keating's manual is his willingness to be racially inclusive with his figures. In lab 8: The Endospore Stain, Keating uses a visual diagram of the steps involved in the staining method. Within this figure, the individual performing the stain is shown with dark skin. In all my years of schooling I have never seen a diagram or figure with a skin tone like mine. Even with it just being a figure, it erased all doubt of my abilities as a scientist from my mind. This small bit of representation is something that should be recognized and acknowledged by all that review this work. I strongly applaud Keating for his forward and inclusive thinking. All images and figures included in the manual are clear, visually appealing, and easy to interpret/follow. The quality of many images and figures in this manual can be compared to those found in a standard textbook, something I find extremely impressive for a lab manual. Once again, Keating goes the extra mile to provide a manual that is engaging, inclusive and student-friendly.

CONCLUSION

In conclusion, Keating's manual goes above and beyond what is expected from a laboratory manual. He provides substantial information for each laboratory procedure in a way that is easy to understand and does not feel overwhelming. The organization of each laboratory is clear and structured. He also includes visual content that is stimulating and lively. He provides information about himself in the preface of the manual which sheds light on his dedication and authenticity as an author, making the manual a greater object of appreciation. I would recommend this for any microbiology professor to adopt into their curriculum.

The views expressed in this book review do not necessarily reflect the opinions of Fine Focus.

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