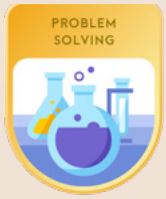
Detecting and Targeting Pathogenic Bacteria Using a CRISPR-Cas13a System



Shada Aborawi, Mina Akbary, Rachel Avileli, Rebecca Avileli, David Basil, Thomas Byrne, Dewuni De Silva, Karen He, Linda He, Mark Lea, Aroma Pageni, Andy Sun, Julien Todd, Katie Vienneau, Natasha Woitte, Elisha Wong, Michelle Wu, Alice Zhang

Winston Churchill High School, Catholic Central High School, Chinook High School, Lethbridge Collegiate Institute (University of Lethbridge), Lethbridge, Alberta, Canada

Reviewed on 11 May 2019; Accepted on 17 June 2019; Published on 28 October 2019

In the coming years, antibiotic-resistant bacteria are expected to become one of the greatest threats to human health. Although antibiotics are credited with improving the health of millions of people since their inception, the overuse of prescription antibiotics has triggered an evolutionary arms race between researchers and pathogens. Additionally, general antibiotics have been shown to cause substantial harm to human microbiomes due to the imbalance of symbiotic and pathogenic bacteria and overuse can result in serious health issues. As such, a modular and specific substitute is sure to replace the current obsolete antibiotics. We propose to use the CRISPR-Cas13a system as a precursor rapid detection and specific targeting system capable of modular design. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats.

This system will ideally target a species-specific RNA transcript in the pathogenic bacteria. Once it interacts with the target, Cas13a becomes activated, initiating non-discriminant cleavage of collateral RNA in the environment. This system has been previously shown for its potential in detection. The system will report if the target sequence from a bacteria is present by a measurable fluorescence change due to collateral cleavage of a signal fluorescent RNA. Furthermore, we plan to partner this system with a thoroughly characterized database of RNA target sequences with specific molecular delivery systems. This will allow us to design multiple systems to target a wide array of bacterial threats. Moreover, by measuring co-culture dynamics through our system we can better translate it to an organism model. This will help us understand the system and predict its effects in real-world events. We hope to enable physicians to improve the accuracy of their diagnosis and assist more people in their recovery from bacterial infection.

Keywords: CRISPR, Cas13a, crDNA, pathogen, endonuclease, antibiotic

Mentors: Sydnee Calhoun, Chris Isaac, Laura Keffer-Wilkes Dia Michailidou-Koupantsis, Luke Saville, Kristi Turton

Watch a video introduction by the authors at http://bit.ly/2Z80c6W

Background

Pathogen detection is becoming an increasing problem in the healthcare industry. Currently, detection methods such as culture tests, imaging scans, and biopsies are used to diagnose pathogens, but many people are suffering from long wait times, occasionally as long as 8 weeks (Boston Children's Hospital). A simple urine culture test, a common method used by doctors to diagnose pathogenic infections, can take 1–3 days for results. It is important to be able to identify and treat infections early in order to avoid sepsis, worsening symptoms, and further complications. To increase the likelihood of early treatment of a pathogen, empiric antibiotics are often administered (Claridge et al. 2010). Empiric or broad-spectrum antibiotics are the use of antibiotics that will likely treat a particular infection prescribed based off of symptoms demonstrated by the patient, but are often found to be ineffective. Antibiotic resistance is becoming a major healthcare concern and it is becoming increasingly difficult to treat and control the spread of infections. According to the Centers for Disease Control and Prevention, one in three antibiotic prescriptions are unnecessary (CDC 2016). Likewise, according to a report by the Organisation for Economic Co-operation and Development, an estimated 404 Canadians die each year due to eight strains of antibiotic resistant bacteria (OECD 2018).

The objective of our project is to create an efficient and rapid detection system and sensitive targeting method to eradicate pathogenic bacteria that helps in restoring the natural microbiome. This will also aid in decreasing the issue of overprescribing antibiotics and will improve point of care testing. When looking for a system to satisfy these criteria, CRISPR-Cas systems have shown success in genome editing. CRISPR is a defense system that some bacteria use to protect themselves against attacks from viruses. CRISPR can be used for genome editing, using the CRISPR-Associated protein Cas9. However, for our system, we are utilizing a different Cas protein called Cas13a, which is an enzyme used to target and cleave RNA. Applications such as SHERLOCK (Specific High-sensitivity Enzymatic Reporter UnLOCKing) created by the Gootenberg group (Gootenberg et al. 2017) show the potential of a Cas13a system in diagnostics. The SHERLOCK system uses a Cas13a detection system combined with the isothermal amplification of nucleic acid sequences. Isothermal amplification uses constant low-temperature reactions to eliminate the need for the expensive equipment required for standard polymerase chain reactions. This system became a portable diagnostic tool for situations where extensive equipment could not be accessed. Using this information, a CRISPR-Cas13a system became the path we decided to take for our system.

To further understand this system, each element of the system will be explained. CRISPR is a new and unique method that carries out genome editing. CRISPR permits scientists to easily edit RNA by creating breaks in specific locations in an RNA strand, which is known as cleaving. Our system employs CRISPR-Cas13a, the RNA sequence of a specific pathogenic bacteria and RNA Mango. RNA Mango has been used in imaging technology due to its fluorescent properties. These fluorescent properties make it a very useful test indicator in our project. CRISPR is being used to specifically cleave the RNA of the targeted pathogen. The recognition of the RNA sequence belonging to the pathogenic bacteria activates Cas13a to randomly cleave all surrounding RNA. When our Cas13a is used in our paper strip diagnosis test, there will also be RNA Mango present. As a result of the collateral effect, RNA Mango will be cut, and this cleavage will break the bond between the RNA Mango aptamer and the fluorophore, thiazole orange (TO1-Biotin), causing color loss. This will indicate a positive test result. Upon insertion of this system into the corresponding bacterial cell, Cas13a would only cleave the RNA of the desired pathogen. This is a critical step in the functioning of our project and would be extremely helpful to those employed in the science field. The cleavage of the desired RNA sequences by CRISPR-Cas13a indicates the presence of pathogenic bacteria and can be used to efficiently eliminate a pathogen from the body.

The proposed detection system would involve the enzyme Cas13a, CRISPR RNA and collateral signal RNA Mango dehydrated on paper test strips. An RNA sequence that would correspond to the bacterial infection we want to detect will be encoded in the CRISPR RNA, allowing Cas13a to associate and form a ribonucleoprotein complex. Upon being exposed to the specific pathogenic RNA sequence, the system would be activated causing the Cas13a to cleave any RNA within its proximity including that of the RNA Mango. The cleaving of the RNA Mango should result in a color change on the paper test strip. Theoretically, the same system could be used to target a pathogenic infection. Due to the specificity of the system, the Cas13a system would only cleave the RNA found within the particular pathogenic bacterial cell, therefore killing only the bacteria and preserving the other elements within the microbiome that would normally be affected by current antibiotics. Protecting the natural microbiome would be beneficial and improve the overall health of patients as we depend on our microbiome to carry out vital functions such as food digestion, production of vitamins, immune system regulation, and protection against other disease-causing bacteria.

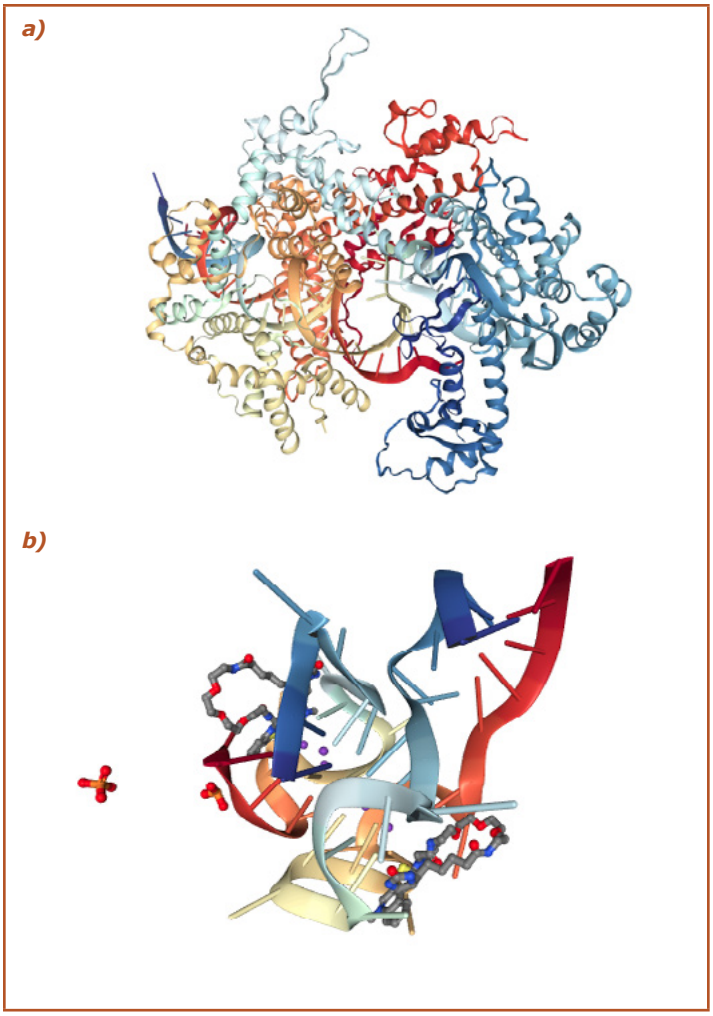


Figure 1. ***a)*** *Crystal structure of the LbuCas13a-crRNA-target RNA ternary complex.* ***b)*** *Co-crystal structure of RNA Mango. (Crystal Structure 2019).*

System Level

Detection System

There are two different aspects to our project: a pathogen detection system and a therapeutic system. The first system of our project is a simple, specific, and effective way to detect pathogens. Our system will use CRISPR/Cas13a along with a fluorescent-tagged RNA called RNA Mango, both of which will be lyophilized (freeze-dried) onto a bioactive paper strip. We will engineer our Cas13a CRISPR RNA (crRNA) to recognize a specific RNA sequence unique to the pathogen of interest. When the crRNA recognizes the sequence, it sets off a reaction referred to as a "collateral effect," whereby all surrounding RNA is cleaved by the Cas13a enzyme. When the Cas13a cleaves the RNA Mango, there is a significant loss of color on the test strip, indicating the presence of the specified pathogen. RNA Mango is a fluorescent-tagged RNA that is a vibrant orange color due to thiazole.

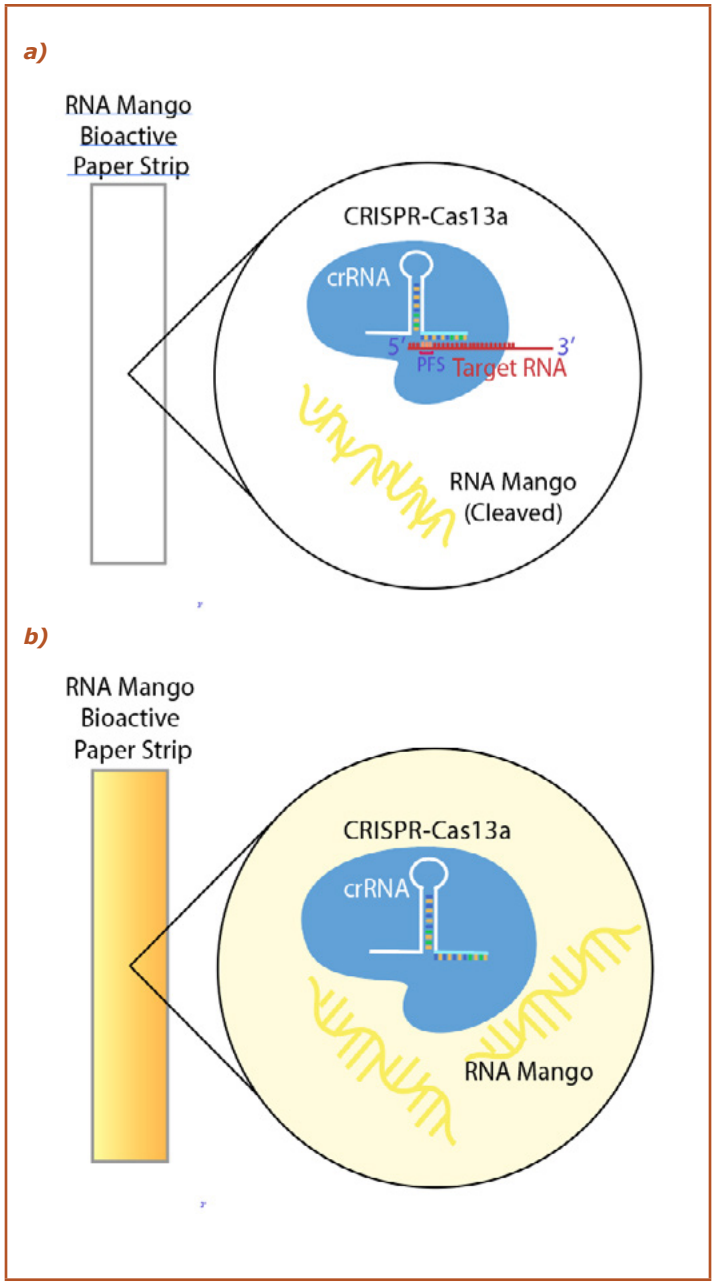


Figure 2. ***a)*** *The diagnostic system including a test strip coated with the RNA Mango, this strip indicates a negative result for a pathogen as the RNA Mango is not cleaved and the color remains.* ***b)*** *This strip indicates a positive result. The target RNA sequence is present on the strip, the CRISPR-Cas13a complex recognizes the sequence and cleavage occurs, resulting in a loss of color on the test strip.*

Targeting System

The second portion of our project utilizes the Cas13a protein as an antimicrobial therapeutic. In order to achieve a targeted delivery of the CRISPR Cas13a construct, we will use a phagemid delivery system. A recombinant plasmid that contains the Cas13a CRISPR construct will be introduced inside the empty bacteriophage. This system of delivery will allow for

us to carry out the therapeutic aspect of our project. Our engineered bacteriophage must have a broad host range for it to be effective in targeting a pathogen. Every bacterium in a specific area will be infected by the phagemid system, but only those containing the target recognition sequence will cause the system to activate. The phagemid system incorporates delivery of our construct that is specific to bacterial strains containing the target sequence.

As such, a modular and specific substitute is sure to replace the forthcoming obsolete antibiotics. We propose to use the CRISPR-Cas13a system as a precursor rapid detection and specific targeting system capable of modular design. This system will ideally target a species-specific RNA transcript in the pathogenic bacteria. Once it interacts with the target, Cas13a becomes activated, initiating non-discriminant cleavage of collateral RNA in the environment resulting in the death of the pathogen (Sven 2019). Based on our knowledge of CRISPR Cas13a we know that it targets and cleaves specific RNA sequences, however we would need to do experiments to learn how rapid our Cas13a enzyme is at cleaving.

Device Level

Pathogenic Bacteria

When determining which pathogenic bacteria to target, we wanted to fulfill two main criteria. Firstly, is the current diagnosis of the infection time-consuming? Our system focuses on fast and efficient diagnosis, and therefore, it would be most beneficial for use with infections containing long diagnostic procedures. Secondly, are the current treatments for the pathogen broad-spectrum antibiotics? Our system is most useful for infections currently treated using general antibiotics, as it eliminates a specific bacterial strain, preventing any side effects associated with broad-spectrum antibiotics. From our research, we decided to target *Staphylococcus aureus* and *Clostridium difficile*, with *Escherichia coli* as our proof of concept.

Staphylococcus aureus

Staphylococcus aureus is both a commensal and pathogenic bacteria. Approximately 30% of the human population carry *S. aureus* as part of their normal microbiomes. However, it is also a leading cause of various infections, such as bacteremia and infective endocarditis (Tong et al. 2015). *S. aureus* infections have generally increased in occurrence over time, particularly methicillin-resistant *Staphylococcus aureus* (MRSA). In fact, the rate of MRSA bacteremia infections in Quebec, Canada increased from 0 per 100 000 person-years to 7.4 per 100 000 person-years from 1995–2005 (Burmeister 2015). *Staphylococcus* infections are a major concern in our world today, especially due to their ability to resist antibiotics through horizontal gene transfer, the lateral transmission of genetic information between organisms (American Association for Clinical Chemistry 2015). The current diagnosis process of *S. aureus* involves laboratory culture of various bodily samples, depending on the site of infection. Such samples include blood and sputum, which can take from 24 to 48 hours (Drug Bank 2019). The duration of time spent before result identification may lead to a worsened state of disease and the prescription of general antibiotics, which may eliminate beneficial bacteria from the healthy gut microbiome. Following the diagnosis of *S. aureus*, a more specifically targeting antibiotic may be necessary, prolonging antibiotic treatment. Extensive antibiotic usage has been exhibited to display many disrupting qualities on human health, which is explicitly demonstrated by two antibiotic groups used to treat *S. aureus*: vancomycin and carbapenems.

Today, *S. aureus* infections are treated using antibiotics, such as vancomycin and its derivatives, carbapenems, cephalosporins, nafcillin or related antibiotics, and sulfa drugs. However, these anti-MRSA β-lactam antibiotics are shown to display negative disruptions to the intestinal microbiomes, leading to a plethora of other ailments. The drug vancomycin is a clear example. Vancomycin is a branched tricyclic glycosylated nonribosomal peptide, formed by the fermentation of *Amycolatopsis orientalis*. It mainly targets gram-positive bacteria by inhibiting bacterial cell wall biosynthesis and altering both RNA synthesis and bacterial cell wall permeability (Drug Bank 2019).

Vancomycin has been identified as active against strains of *Listeria monocytogenes*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Actinomyces* species, and *Lactobacillus* species (Drug Bank 2019). It has subsequently been shown to negatively impact the gut microbiome by the depletion of operational taxonomic units (OTUs) of the Bacteriodetes phylum, and the expansion of species of the Proteobacteria phylum, such as *Klebsiella*; an increase in such species are associated with disease (Isaac et al. 2016). Moreover, the prolonged use of vancomycin to treat MRSA infection is demonstrated to cause vancomycin intermediate-resistant *Staphylococcus aureus* (VISA), often ending in treatment failures. As vancomycin is often referred to as a 'last resort' antibiotic, the development of VISA poses a great threat (Gardete and Tomasz 2014).

Another treatment for S. aureus infections are carbapenems. Carbapenems are β lactam antibiotics that are extremely broad-spectrum. Some of the bacteria that it targets include *Streptococci, Staphylococci, Entero-*

bacteriaceae, and *Haemophilus influenza* (Merck Manual 2018). The broad-spectrum targeting nature of carbapenems create harmful microbial imbalances within the human microbiome. For example, it has been shown that broad-spectrum antibiotics reduce the number of various immune cell populations, such as macrophages and T lymphocytes, therefore weakening the strength of the immune system (Ekmekciu et al. 2017). Carbapenems are also strongly associated with antibiotic resistance. It has been shown that antibiotic-resistant strains, particularly vancomycin-resistant *Enterobacteriaceae*, ciprofloxacin-resistant P. *aeruginosa* and MRSA are identified in 14 cases per 1000 antibiotic days (Bhalodi et al. 2019). Additionally, the excessive use of carbapenems has resulted in carbapenem-resistant bacteria, as these bacteria start to produce carbapenemase. This is extremely concerning because, similarly to vancomycin, carbapenems are used as a last resort for multidrug-resistant gram-negative bacteria; the inactivation of "last resort" drugs could cause a situation similar to the pre-antibiotic era. Taken together, the current, slow diagnostic methods and microbiome-disturbing treatments for *S. aureus* infection demonstrate the necessity for a new diagnostic and therapeutic approaches.

Clostridium difficile

Clostridium difficile is a gram-positive, spore-forming bacteria responsible for the formation of pseudomembranous colitis (PMC) and inpatient cases of antibiotic-associated diarrhea (AAD). Some of the common risk factors for *C. difficile* infection include the frequent use of antibiotics, older age, and diabetes mellitus (Oka et al. 2012). *C. difficile* infection is a major concern in Canada; in 2012, it was estimated that 37 932 Canadians suffered from the illness, and it is a growing concern within many other countries as well (Katz et al. 2018).

In fact, there has been an increasing rate of *C. difficile* infection populations who are not considered at high risk for infection, such as in children without a history of antibiotic use. The diagnosis procedure of *C. difficile* includes sending stool samples to the lab. Nucleic acid amplification test (NAAT) is one method of sample analysis. Although it can take a minimum of one hour for results to become available, this method of testing can produce false results due to its inability to distinguish *C. difficile* infection from asymptomatic carriers. This has resulted in the over-prescription of antibiotics. Another test used to diagnose *C. difficile* is anaerobic bacterial culture; however, this process is time-consuming (Lamont et al. 2017).

Similarly to S. aureus, the diagnosis of C. *difficile* is time-consuming, and the treatment is broad-spectrum antibiotics, such as vancomycin or metronidazole. The use of these broad-spectrum antibiotics poses many threats, such as immunodeficiency. In addition, using these antibiotics has been shown to be slightly ineffective, as recurrent cases of C. *difficile* associated diseases have rates of 10-35% or higher. Therefore, the creation of a quicker, more accurate test is desirable for C. difficile infections (Oka et al. 2012).

Escherichia coli (proof of concept):

Pathogenic strains of *E. coli*, such as *E. coli* O157: H7, is another potential target for our proposed detection system. In this case, however, rather than serving as a diagnostic tool, the system can instead be used in a screening assay to detect contamination by pathogenic *E. coli* in food items and water for consumption and recreational uses. Current methods of detection for *E. coli* O157:H7 as recommended by regulatory and public health agencies depend largely on conventional culturing (Tortorello and Stewart 1998). Although newer methods of rapid detection using real-time polymerase chain reaction had been developed, they still require at least six hours of enriching the test sample (i.e. raw meat) in a universal enrichment broth (UPB) to facilitate bacteria growth, and they tend to have a high lower detection limit (Piskernik et al. 2010). Successful implementation of our system can reduce the time needed for detection, increase specificity, and decrease the lower detection limit.

Targeting pathogenic *E. coli* inside the human gastrointestinal tract will present a greater challenge, as non-pathogenic strains of *E. coli* constitute a normal part of the gut microbiome of a healthy human individual, leading to increased risks of false positives. Fortunately, a gene has been previously identified that characterizes the pathogenic strain and is not found in other strains. The *stx*1 and *stx*2 genes are two genes found exclusively in the pathogenic *E. coli* O157: H7 that are responsible for the production of shiga-like toxins, which lead to various symptoms associated with the infection (Adelman et al. 2014). By engineering the CRISPR RNA of our CRISPR-Cas 13a complex to specifically target the mRNA transcript of *stx*1 or *stx*2, we can ensure that only those strains of *E. coli* with pathogenic effects will be targeted while other naturally-occurring strains in the gut microbiome are spared.

Additionally, we will use *E. coli* in our lab assays as a proof of concept, as *E. coli* is the most extensively-studied and readily-available bacteria species. The *E. coli* strains that will be utilized during our project will be DH5α and BL21(DE3) for genetic manipulation and protein overexpression.

Moreover, the overall design of our system will function by having a paper strip test that will detect pathogenic bacteria present in our body. These paper strips will

Bacterial Infection	Current Diagnosis Options	Sample Type	Quantity of Sample Required	Time for Results (hours)	Costs (dollars CAD)	References
Staphylococcus aureus	Bacterial Culture	Blood	10 mL	24-48	$36.08	Drug Bank 2019
	Bacterial Culture	Stool	1 g	24-48	$14.42 - $105.04	British Columbia Ministry of Health 2019
Clostridium difficile	Nucleic Acid Amplification Test (NAAT)	Blood/Stool/ Tissue	10 mL		$36.08	Adelman et al. 2014
	Bacterial Culture	Stool	1 g	24-48	$14.42 - $105.04	Tenover et al. 2011
Escherichia coli	Real-Time Polymerase Chain Reaction (PCR)	Stool	1 g	48-92	$5.46 - $40.37	Mahony et al. 2004
	Bacterial Culture	Blood	10 mL	48-92	$36.08	British Columbia Ministry of Health 2019

Table 1. *A comparison of various diagnosis options of Staphylococcus aureus, Clostridium difficile, and Escherichia coli. Included are the sample types, sample quantity requirements, time requirements, and costs.*

contain a Cas13a and RNA Mango system. RNA Mango is a strand of RNA with a specific three-dimensional structure, which upon binding to thiazole orange (chemical fluorophore) will become colored and fluoresce. Therefore, when the RNA of the pathogenic bacteria is detected the collateral cleavage will begin, causing the RNA Mango to be cleaved as well. When this occurs, the color that was once present on the strip will be lost, indicating a positive result. If the color is not lost the pathogenic bacteria has not been cleaved and it is a negative result.

Targeting the Bacteria

Phagemid

A potential method to deliver our Cas13a system is by using phagemids. Phagemids are phage-derived, DNA cloning vectors (Qi et al. 2012) that have very efficient transformation properties. Some components of phagemids include the replication origin of a plasmid, the selective marker, the intergenic region, a phage coat protein gene, restriction enzyme recognition sites, DNA encoding a single peptide, and a promoter.

Phagemids would be an efficient method of delivering our system as phagemids are basically phage plasmids (hence the name). This means that our Cas13a system would be able to be enclosed within the phagemid and injected into bacteria in our body. Upon entrance into each bacterial cell, the Cas13a will be inactive until it identifies the RNA sequence corresponding to its crRNA, where it will initiate cell death through the collateral effect.

Cell Penetrating Peptides

Cell-penetrating peptides (CPPs) are another potential mode of delivery for our Cas13a system. CPPs are short peptides from 8–30 amino acids in length and are most often hydrophobic and cationic (Watson et al. 2017). They deliver functional biomolecules through cell membranes, such as proteins, peptides and siRNA. There are three main classes of cell-penetrating peptides: protein derivatives, chimeric peptides and synthetic peptides (Bachara and Sagan 2013).

CPPs' ability to deliver proteins demonstrates its potential to be used as a delivery method for our system. Upon the administration of CPPs containing our system to the patient, the CPPs would theoretically penetrate a broad range of cells with our engineered CRISPR protein. If Cas13a detects the RNA sequence of interest, collateral cleavage will cause the pathogen to die. However,

CPPs pose multiple problems when used with our system. For one, CPPs are structurally similar to antimicrobial peptides, as demonstrated by an experiment performed by Stockholm University (Nekhotiaeva et al. 2004). The CPP TP10 inhibited the growth of *S. aureus* and *Candida albicans*. The antimicrobial effects of CPPs are not favourable, since they may affect the results of our paperstrip detection system. For example, the usage of TP10 would potentially cause a decrease in S. aureus, resulting in an inaccurate measure of color loss. Additionally, the killing of various beneficial organisms within humans could create other issues in terms of microbiome health-- problems that we are attempting to address with our own system. Therefore, CPPs may be considered as a delivery mode for our system, although their antimicrobial nature poses several concerns in terms of its compatibility.

Parts Level

CRISPR

For our system to operate efficiently and effectively we chose to use Cas13a instead of Cas9 for a variety of reasons. The main difference between Cas9 and Cas13a is that Cas9 targets DNA in a genome, whereas Cas13a targets RNA. Targeting RNA is more beneficial for our project as it is more abundant, allowing it to be easily targeted. Not only is Cas13a useful in targeting RNA, but it also has a property which creates collateral cleavage. Once the Cas13a detects the RNA of the target bacteria, it will undergo the collateral effect in which all of the surrounding RNA will be cleaved. When the RNA of the pathogenic bacteria is detected, the collateral cleavage will begin, causing surrounding RNA Mango to be cleaved as well. This will result in an absence of color that is crucial in the detection aspect of our system (Azvolinsky 2019).

Cas13a Protein (BBa_K2306012)

We have chosen to investigate Cas13a enzymes from four different bacterial species, including LshCas13a, LbuCas13a, LwaCas13a, and LbaCas13a. Each different variation of the Cas13a protein has unique strengths and weaknesses that have been previously researched. Abudayyeh et al. (2017) states that *Leptotrichia wadei* (LwaCas13a) was the most effective of the fifteen orthologs in *E. coli.* It is as effective as RNA interference (RNAi) and a more specific RNA targeting option (Abudayyeh et al 2017). This improved specificity would make it ideal for our system. However, our team would like to conduct further research as well as our own laboratory experiments to determine which type of Cas13a would be best suited to our system.

CRISPR RNA (crRNA)

In order for cleavage of RNA to occur, a crRNA strand is used with the Cas13a protein, which has a uracil-rich stem-loop conformation as well as a recognition sequence for the target RNA. The crRNA allows for the recognition of the protospacer adjacent motif (PFS) of the target RNA. We are able to order sequences and append them to the crRNA depending on our application. The protospacer adjacent motif (PFS) of the target RNA must be compatible with the crRNA sequence for recognition and subsequent cleavage, Higher Eukaryotes and prokaryotes nucleotide binding (HEPN) domains on the Cas13a proteins allow for ribonuclease activity. The RNA binding domains are activated when the complementary crRNA binds to the target, and Cas13a is triggered to cleave RNA by activating the HEPN domains. The RNA molecule that activated the complex, as well as any other RNA in the vicinity, will be cleaved by CRISPR-Cas13a.

RNA Mango

RNA Mango is a high-affinity RNA aptamer that binds to the fluorophore thiazole orange with nanomolar affinity, with KD ≈ 3nM. On its own, thiazole orange (TO1), an asymmetric cyanine fluorophore, has very little fluorescence; however, when bound to the RNA Mango aptamer, the system undergoes a 1100-fold increase in fluorescence. The fluorescence of thiazole orange occurs when the monomethine bridge connecting the two heterocycles rigidifies by insertion into double-stranded helical nucleic acids, securing the thiazole orange in place within the aptamer (Dolgosheinna et al. 2015).

Incorporating RNA Mango into our paper strip detection system provides clear identification of pathogenic presence. In comparison to other fluorescence techniques, RNA Mango is almost 2 times greater in fluorescence to the RNA Spinach aptamer, therefore demonstrating its higher fluorescence efficiency. Upon recognition of the specific pathogenic bacterial RNA of the target, the engineered Cas13a will initiate the collateral RNA cleavage effect, where surrounding RNA is randomly cut—including the RNA Mango aptamer and TO1-Biotin Complex. Its cleavage will result in the detachment of the aptamer from the fluorophore, wherein color loss will occur. By analyzing the fluorescence changes of RNA Mango, the presence of a pathogen can be visually detected on our paper strips.

Phagemid System

One of our proposed methods of delivery involves usage of a broad-spectrum bacteriophage (bacteria infecting virus) that inserts our CRISPR-Cas13a system into a large range of bacterial cells. All viral genes will be

removed from the phage, thereby inhibiting cell lysis. Only cells containing the recognition sequence matching our engineered crRNA sequence will activate the HEPN domains of the CRISPR-Cas13a complex. The pathogenic bacteria will then undergo cell death by the collateral cleavage effect from the CRISPR system, whereas other bacterial cells will remain. There are multiple options of broad-range phages that we have looked at for our delivery system (Qi et al. 2012).

Phage for Methicillin-Resistant Staphylococcus aureus

SLPW is a lytic phage with a broad host range that infects *Staphylococcus aureus*, a pathogen that is becoming increasingly resistant to drugs. This bacteriophage has been shown to maintain stability at temperatures up to 45º C, and in a pH range of 2–12 (Wang et al. 2016).

Phage for Clostridium difficile

Bacteriophages that have a broad host range including *C. difficile* have yet to be characterized due to the difficulty in culturing the pathogen. Experimentation on potential phages that are specific to *C. difficile* reveals that a phage ΦHN10 successfully attached to the cell wall. Stability tests were also conducted on the phages, where they were found to be stable within the pH range of 5 to 10. Thermal stability tests showed the phages to be stable between 50 and 60 C (Phothichaisri et al. 2018).

Second Phage for C. difficile

C. difficile myovirus phages have been characterized to target *C. difficile* pathogens in a 4-phage cocktail. CDHM1, CDHM2, CDHM5, and CDHM6 were shown to interact with and infect C. difficile within the human microbiome, indicating their potential as phagemids (Nale et al. 2018).

Proof of Concept Phage

For testing purposes, we plan on using a phage that infects *E. coli* as it is a well-characterized organism. The T4 bacteriophage is a model phage for proof of concept engineering (Ferenc 2019).

Fluorescent Proteins

In the color assay portion of our system, we will be using three different fluorescent proteins. We have chosen these specific proteins as they are easily accessible and can be easily detected by our instruments.

Color Assay

As a proof of concept, we will aim to produce three different *E. coli* strains, each one producing a different fluorescent protein (GFP, BFP or RFP). Using crDNA specific to each of the individual protein mRNAs, we can target only cells producing the desired protein (and mRNA). Cells will be transformed with our plasmid constructs, one plasmid that will express the fluorescent protein, one plasmid that will express the crDNA, and one plasmid that will express the Cas13a protein. The plasmids containing the CRISPR components will be under the control of the T7 promoter, thus they will only express when we turn them on. The fluorescent proteins will be constitutively expressed, meaning that they will always be turned on. If we have RFP and the crDNA was designed to target RFP, then no expression of RFP would be observed because the mRNA has degraded. Likewise, if we had RFP and the crDNA-GFP, nothing would occur as the expressed color will still remain red.

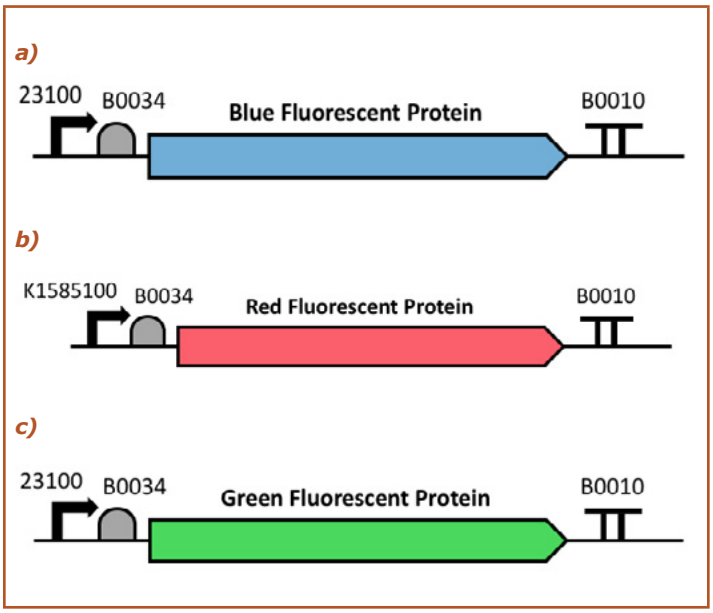


Figure 3. ***A)*** *BFP: Blue fluorescent proteins produce high blue fluorescence color. When GFP is mutated at different amino acid groups it can change to fluoresce in the blue spectrum. This fluorescent protein will also be used as a marker in our system (Hoffman and Conn 2012).* ***B)*** *RFP: Red fluorescent protein emits red fluorescent light when it is used in detection. The use of RFP in our system will help us obtain results for the colorimetric assay (Ormo et al. 1996).* ***C)*** *GFP: Green fluorescent protein is a protein found in Aquorea victoria jellyfish that exhibits green fluorescence when exposed to light. This protein is coded for by a single gene. We will be using GFP in the therapeutic component of our system in the color assay, as a marker protein (Kermers et al. 2011).*

Safety

Safety is imperative when working with Cas13a because if an unintended target is identified as the correct target it could be problematic. While a false positive such as this occurring is the greatest concern when working with

CRISPR, many still have issues about ethical issues regarding gene editing. In this section, we will discuss the problems we may encounter and the steps we will take to prevent the issues from occurring.

One of the major threats is the possibility of RNA being degraded or edited at a site other than the intended target site. These 'off-target' effects can lead to unintended mutations, and as such are a major concern in dealing with CRISPR (44). Since DNA and RNA are billions of combinations long, the chance of a false positive occurring can be greater than 50% when using CRISPR (Zhang et al. 2015). While this is most common in amplified genomic regions, or regions in which the amount of DNA in the chromosome has increased, (Munoz et al. 2016) within cancer cells we can still safeguard against false positives by targeting a longer strand of single guide RNA (sgRNA) at the 5' end, as this decreases the chance 5000 fold in some cases (Redman et al. 2016). The sgRNA is used to direct the Cas protein towards the targeted nucleic acid strand once it is detected (Ishida et al. 2016). Another way to prevent false positives is to decrease the length of the sgRNA strand we are targeting at the 3' end of the sequence (Weisberger 2019). While these methods will reduce the chance of false positives, there is still a small chance of them occurring.

Another method used to reduce the chance of off-target effects in CRISPR is targeting a segment that has a very unique sequence, due to the tendency of CRISPR to target sites even with three to five mismatched pairs (Chew 2017). We recognize this importance and will create a database of a number of said sequences in order to make it easier to find these unique ones. This program would reference this database in an attempt to match with existing sequences or to find similar, different sequences. The ability to search for these unique sequences in pathogenic bacteria will make proper targeting more accessible to us and others.

While the usage of CRISPR is still hotly debated, and though it may be a buzzword, most of the conversations revolve around whether it is right to edit human DNA. As shown by the backlash against Chinese scientist Jiankui He, it is argued that it is not acceptable to edit the genes of a human, even for a good cause (Chew 2016). Some people are opposed to the use of CRISPR for ethical reasons, however, this represents a very small number of people and this is not a very mainstream belief. Further, our system does not use CRISPR for gene editing purposes. Thus while we do believe that the discussion about the morality of gene editing is important, very few people are strongly opposed to the use of CRISPR in this context, to detect infections. It is for these reasons that we believe the ethicacy of our project is not in question.

Another danger in working with CRISPR is the possibility of immune responses from the body. Potential defense systems include inflammation, antibody induction, and cell death as a result of T-Cell-mediated cytotoxicity (Kosicki et al. 2018). The most commonly used source of Cas proteins is found in bacteria which may cause diseases in humans. It has been found, likely as a consequence, that there can often be immune responses to them. When mice were given adenovirus-delivered spCas9, increases in the presence of cas9 antibodies were observed (Kim et al. 2018). Indeed, both cellular and humoral immune responses have been detected (Sack and Herzog 2009). It has been shown, as well, that *in vitro* transcribed guide RNAs have set off immune responses in human cells that may lead to 80% cell death (Sadelain 2004).

An additional concern comes in the form of what delivery system is chosen to carry CRISPR to the cells. An easy choice of efficient, viral vectors, unfortunately involves some safety concerns due to a potential immune response from the human body (Hosseinidoust 2017), and mutagenesis, even leading to the growth of tumors (Hosseinidoust 2017). Additionally, viral vectors often require the presence of a helper virus for replication. This helper virus further increases the chance of contamination. To allow for many of the advantages, and to avoid some of the safety concerns of viral delivery, our system will use phage-based delivery specific for bacteria cells. Bacteriophages naturally exist in the human body, and thus are unable to invade mammalian cells, but have evolved to infect bacterial cells with high specificity (Hosseinidoust 2017).

Discussions

The current methods of pathogen detection and treatment are limiting and thus, the development of a new system is necessary. Pathogen detection is used for a variety of reasons, one of which includes testing the quality of drinking water and food.Some of the methods used to detect the microbial pathogens in wastewater and drinking water include culture, PCR (Polymerase Chain Reaction), and microscopy (Toze 1999). These methods are expensive, time-consuming, and may result in false positives, demonstrating their lack of efficiency. Another use of pathogen detection is for human health; however, the current detection methods of bacterial infections such as *Staphylococcus aureus* and *Clostridium difficile* are time-consuming, as they often require culturing the samples before a firm diagnosis is made (Singh et al. 2018). Moreover, the overexposure of antibiotics in today's society-- particularly general antibiotics-- has resulted in the formation of antibiotic-resistant bacterial strains, rendering the antibiotics obsolete.

Our system of detecting pathogens using synthetic biology and CRISPR Cas13a has the potential to solve the aforementioned problems. The time-consuming pathogen tests currently used for both water and human testing can be addressed more efficiently using our proposed bioactive paper strips, providing immediate results. In regards to the nonspecific nature of certain antibiotics used as treatment, our specific targeting system has the potential to solve that issue. Due to the Cas13a only cleaving when matched with the RNA of interest, only the exact strain of target bacteria will be killed. This will prevent overexposure of the microbiome to antibiotics. As our system has not been used to target pathogens previously, it is less likely that the bacteria will gain a resistance to our therapeutic in a short period of time. This should provide a solution when antibiotics are unable to work due to antibiotic-resistant bacteria and will not contribute to the growth of antibiotic-resistant bacteria.

Another potential application of our system could be a pathogen detection method for astronauts. Bacterial culture is very difficult to perform in space, yet a study done by researchers from NASA's jet propulsion laboratory found several species of bacteria that would have a great chance of developing antibiotic resistance on the International Space Station. This demonstrates the requirement for a simple and effective test in space for various pathogens.

Our system can help address these issues of antibiotic resistance on space missions on a smaller scale, currently, and on a larger scale, for future space travel and benefit to people.

Confined environments, such as those in space stations, can foster disease and bacteria.Our test strips could be used for astronauts (testing bodily fluid samples), and surfaces (discouraging the spread of illness and taking preventative measures). Currently, there are some methods used to prevent the growth of bacterial pathogens; for example, AGXX®, a bioactive contact catalyst that kills pathogenic bacteria upon contact (Sobisch et al. 2019). However, there are few systems available for detecting the bacteria, which is where our system can be implemented.

Some challenges that may need to be overcome is if the Cas13a doesn't detect the RNA target, as the activation of Cas13a will not occur. Furthermore, another problem that could occur is if the RNA Mango on our paper strip test does not decrease in color intensity. This is an issue, as the results of the detection system will not be visible, and therefore an inaccurate result will occur. It may also be difficult to find an RNA segment that is unique to the specified pathogen, which may compromise the accuracy of our project. If the RNA segment for the therapeutic part of our system is shared with, for example, one of our body cells, it may damage our microbiome, or for the diagnostic part, if in our bodily fluid, it may create false positives. Additionally, if the sequence is too similar to sequences shared by our microbiome, it increases the potential of off-target effects. To avoid this, we plan to create a software to act as a database of genomically-unique sequences.

Another issue that we need to face would be the way in which we administer the CRISPR Cas13a system into a human body, and how the targeted bacteria will accept the Cas13a. Cas13a is a relatively new discovery and so far it is not as well-characterized as other Cas proteins. As mentioned above, certain delivery systems such as viral vectors which are more efficient also have drawbacks involving safety and the immune system. Our proposed phagemid delivery is, in many ways, a happy medium.

To ensure that our system will target the pathogen efficiently, we will use a fluorescent assay to measure cleavage of RNA. This test will determine the time taken to cleave the RNA and how long it will take to see the visible results of the color change. This test will be beneficial as our system can then be tested to see if it will be an efficient solution for an ongoing issue in society. Additionally, we will be conducting a colorimetric assay to show and test the specificity of the targeted gene and how well it works. If the correct protein is targeted, the protein will stop being produced which will cause a loss of color, thus indicating a positive result.

Acknowledgements

The Lethbridge High School iGEM team acknowledges the support of the geekstarter / Mindfuel Program, the University of Lethbridge Department of Chemistry and Biochemistry, and team advisors Dr. Laura Keffer-Wilkes, Chris Isaac, Luke Saville, Dia Michailidou-Koupantsis, Kristi Turton, and Sydnee Calhoun.

References

Abudayyeh O., Gootenberg J., Essletzbichler P., Han S., Joung J., Belanto J., Verdine V., Cox D., Kellner M., Regev A., Lander E., Voytas D., Ting A., Zhang F. RNA targeting with CRISPR-Cas13a. National Center for Biotechnology Information. 2017;550(7675):280-284.

Adelman MW, Kurbatova E, Wang YF, Leonard MK, White N, et al. Cost Analysis of a Nucleic Acid Amplification Test in the Diagnosis of Pulmonary Tuberculosis at an Urban Hospital with a High Prevalence of TB/HIV. PLOS ONE. 2014;9(7): e100649. https://bit.ly/2MlJHvw

American Association for Clinical Chemistry. Staph Infections and Methicillin-Resistant Staphylococcus aureus. (2016, November 30th). Retrieved 2019 May 1 from: https://bit.ly/31RlkfR

Azvolinsky A., CRISPR System Targets RNA in Mammalian Cells. The Scientist. Retrieved 2019 May 1 from: https://bit.ly/2SKjDAu

Bechara C., Sagan S. Cell-penetrating peptides: 20 years later, where do we stand? 2013;587(12):1693-1702.

Bhalodi A. A., Engelen T. S., Virk H. S., Wiersinga W. J. Impact of antimicrobial therapy on the gut microbiome. Journal of Antimicrobial Chemotherapy. 2019 74(1): i6-i15.

Boston Children's Hospital. Spinal Tap Recovery Time [Internet]. Retrieved from: https://on.bchil.org/2On7cXP

British Columbia Ministry of Health (2015, October 1). Schedule of Fees. Retrieved 2019 May 31 from: https://bit.ly/2AHLHZQ

British Columbia Ministry of Health (2015, October 1). Schedule of Fees. Retrieved 2019 May 31 from: https://bit.ly/2AHLHZQ

Burmeister, A. R. 2015. Horizontal Gene Transfer. Evolution, Medicine and Public Health.2015;1:193-194.

CDC. 2016. CDC: 1 in 3 antibiotic prescriptions unnecessary [Internet]. Retrieved 2019 from: https://bit.ly/2mwqqcj

Chew WL. Immunity to CRISPR Cas9 and Cas12a Therapeutics. Wiley Interdisciplinary Reviews: Systems Biology and Medicine. 2017;10(1).

Chew WL., Tabebordbar M., Cheng JK., Mali P., Wu EY., Ng AH., Zhu K., Wagers AJ., Church GM. A multifunctional AAV-CRISPR-Cas9 and its host response. Nature Methods 2016;13(10):868-874.

Cho SW., Kim S., Kim Y., Kweon J., Kim HS., BAe S., Kim JS. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Research. 2014;24(1):132-141.

Claridge JA, Pang P, Leukhardt WH, Golob JF, Carter JW, Fadlalla AM. Critical Analysis of Empiric Antibiotic Utilization: Establishing Benchmarks. 2010;(2):125–131.

Crystal structure of LbuCas13a-crRNA-target RNA ternary complex, Co-crystal structure of the fluorogenic RNA Mango [Internet]. Retrieved 2019 from: https://www.rcsb.org/

Drug Bank. Vancomycin [Internet]. Retrieved 2019 May 1 from: https://www.drugbank.ca/drugs/DB00512 , May 2nd, 2019.

Ekmekciu I., Klitzing E., Fiebiger U., Escher U., Neumann C., Bacher P., Scheffold A., Kühl A. A., Bereswill S., Heimesaat M. M. Immune Responses to Broad-Spectrum Antibiotic Treatment and Fecal Microbiota Transplantation in Mice. Frontiers in Immunology. 2017;8:397.

Elena V. Dolgosheina, Sunny C. Y. Jeng, Shanker Shyam S. Panchapakesan, Razvan Cojocaru, Patrick S. K. Chen, Peter D. Wilson, Nancy Hawkins, Paul A. Wiggins, Peter J. Unrau. RNA Mango Aptamer-Fluorophore: A Bright, High-Affinity Complex for RNA Labeling and Tracking. 2015. Retrieved 2019 from: https://bit.ly/2AMr7Ye

Farahmandfar M., Moori-Bakhtiari N., Zarei M. Comparison of two methods for detection of E. coli O157H7 in unpasteurized milk. Iranian Journal of Microbiology. 2016;8(5):282-287.

Ferenc M. Plasmids 101: Common Lab E. coli Strains. Addgene's Blog. Retrieved May 4, 2019 from: https://bit.ly/2LOwtZn

Gardete S., Tomasz A. Mechanisms of vancomycin resistance in Staphylococcus aureus. The Journal of Clinical Investigation. 2014;124(7): 2836-2840.

Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, Verdine V, Donghia B, Daringer NM, Freije CA, Myhrvold C, Bhattacharayya RP, Livny J, Regev A, Koonin EV, Hung DT, Sabeti PC, Collins JJ, Zhang F. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science. 2017;356:438–442.

Hoffman RM., Conn PM. Imaging and Spectroscopic Analysis of Living Cells: Imaging Live Cells in Health Disease/Live Cell Imaging in Live Animals with Fluorescent Proteins. Fluorescent Proteins. 2012;506(11):197-224.

Hosseinidoust, Z. Phage-Mediated Gene Therapy, Current Gene Therapy, 2017B;17(2):120-126

Isaac S., Scher J. U., Djukovic A., Jiménez N., Littman D. R., Abramson S. B., Pamer E. G., Ubeda C. Short- and long term effects of oral vancomycin on the human intestinal microbiota. Journal of Antimicrobial Chemotherapy. 2016;72(1):128-136.

Ishida K., Gee P., Hotta A. Minimizing off-Target Mutagenesis Risks Caused by Programmable Nucleases. International Journal of Molecular Science. 2015;6(10):24751-24771.

Katz K. C., Golding G. R., Baekyung K., Pelude L., Amaratunga K. R., Taljaard M., Alexandre S., Collet J., Davis I., Du T., Evans G. A., Frenette C., Gravel D., Hota S., Kibsey P., Langley J. M., Lee B. E., Lemieux C., Longtin Y., Mertz D., Mieusement L. M. D., Minion J., Moore D. L., Mulvey M. R., Richardson S., Science M., Simor A. E., Stagg P., Suh K. N., Taylor G., Wong A., Thampi N. The evolving epidemiology of Clostridium difficile infection in Canadian hospitals during a postepidemic period (2009–2015). The Canadian Medical Association Journal. 2018;190(25):758-765.

Kim S., Koo T., Jee HG., Cho HY., Lee G., Lim DG., Shin HS., Kim JS. CRISPR RNAs trigger innate immune responses in human cells. Genome Research. 2018;28(3):367–373.

Kosicki M., Tomberg K., Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nature Biotechnology. 2018;36(8): 765-771.

Kremers GJ., Gilbert SG., Cranfill PJ., Davidson MW., Piston DW. Science. Fluorescent Proteins at First Glance. J cell Sci. 2011;(124):157-160.

Lamont MD, Kelly MD, Bakken MD. Clostridioides (formerly Clostridium) difficile infection in adults: Clinical manifestations and diagnosis. Post TW, ed. UpToDate [Internet]. Waltham, MA: UpToDate Inc. Retrieved 2017 May 2 from: https://www.uptodate.com

Mahony, J. B., Petrich, A., Louie, L., Song, X., Chong, S., Smieja, M., … Ontario Laboratory Working Group for the Rapid Diagnosis of Emerging Infections. Performance and Cost evaluation of one commercial and six in-house conventional and real-time reverse transcription-pcr assays for detection of severe acute respiratory syndrome coronavirus. Journal of clinical microbiology, 2004;42(4):1471–1476. doi:10.1128/jcm.42.4.1471-1476.2004

Merck Manual. Carbapenems [Internet]. 2018 July. Retrieved 2019 May 1 from: https://mrkmnls.co/321FtzK

Munoz, D., Cassiani, P., Li, L., Billy, E., Korn, J., Jones, M., Golji, J., Ruddy, D., Yu, K., McAllister, G., DeWeck, A., Abramowski, D., Wan, J., Shirley, M., Neshat, S., Rakiec, D., de Beaumont, R., Weber, O., Kauffmann, A., McDonald, E., Keen, N., Hofmann, F., Sellers, W., Shmelzle, T., Stegmeier, F., Schlabach, M. CRISPR Screens Provide a Comprehensive Assessment of Cancer Vulnerabilities but Generate False-Positive Hits for Highly Amplified Genomic Regions. Cancer Discovery. 2016;6(8):900-13.

Nale J. Y., Redgwell T. A., Millard A., Clokie M. R. J. Efficacy of an Optimised Bacteriophage Cocktail to Clear Clostridium difficile in a Batch Fermentation Model. 2018;7(1):

Nekhotiaeva N., Elmquist A., Rajarao G., Hällbrink M., Langel U., Good L. Cell entry and antimicrobial properties of eukaryotic cell-penetrating peptides. Federation of American Societies for Experimental Biology. 2004;18(2):394-396.

OECD. 2018. Stemming the Superbug Tide: Just A Few Dollars More, OECD Health Policy Studies, OECD Publishing, Paris, https://doi.org/10.1787/9789264307599-en.

Oka K., Osaki T., Hanawa T., Kurata S., Okazaki M., Manzoku T., Takahashi M., Tanaka M., Taguchi H., Watanabe T., Inamatsu T., Kamiya S. Molecular and Microbiological Characterization of Clostridium difficile Isolates from Single, Relapse, and Reinfection Cases. Journal of Clinical Microbiology. 2012;50(3):915-921.

Oka K., Osaki T., Hanawa T., Kurata S., Okazaki M., Manzoku T., Takahashi M., Tanaka M., Taguchi H., Watanabe T., Inamatsu T., Kamiya S. Molecular and Microbiological Characterization of Clostridium difficile Isolates from Single, Relapse, and Reinfection Cases. Journal of Clinical Microbiology. 2012;50(3):915-921.

Ormo M.,Cubitt AB., Kallio K., Gross LA., Tsien RY., Remington SJ. Crystal structure of the Aequorea victoria green fluorescent protein. Science. 1996 (273): 1392-1395.

Phothichaisri W, Ounjai P, Phetruen T, Janvilisri T, Khunrae P, Singhakaew S, Wangroongsarb P, Chankhamhaengdecha S. Characterization of Bacteriophages Infecting Clinical Isolates of Clostridium difficile. Front Microbiol. 2018 Jul 31;9:1701. doi: 10.3389/fmicb.2018.01701. PMID: 30108562; PMCID: PMC6079236.

Piskernik S., Klancnik A., Toplak N., Kovač M. Rapid detection of Escherichia coli O157:H7 in food using enrichment and real-time polymerase chain reaction. Journal of Food and Nutrition Research. 2010;49(2):78-84.

Qi H., Lu H., Qiu H., Petrenko V., Liu A. Phagemid Vectors for Phage Display: Properties, Characteristics and Construction. Journal of Molecular Biology. 2012; 417:129-143.

Redman M., King A., Watson C., King D. What is CRISPR/Cas9?. Archives of disease in childhood. Education and practice edition. 2016;101(4):213-215

Sack BK., Herzog RW. Evading the immune response upon in vivo gene therapy with viral vectors. Current Opinions in Molecular Therapy. 2009;.11(5):493-503.

Sadelain, M., Insertional oncogenesis in gene therapy: how much of a risk? Gene Therapy. 2004;11(7):569-573.

Singh N, Bezdan D, Checinska Sielaff A, Wheeler K, Mason C, Venkateswaran K. Multi-drug resistant Enterobacter bugandensis species isolated from the International Space Station and comparative genomic analyses with human pathogenic strains. BMC Microbiology. 2018;18(1).

Sobisch L-Y, Rogowski KM, Fuchs J, Schmieder W, Vaishampayan A, Oles P, et al. Biofilm Forming Antibiotic Resistant Gram-Positive Pathogens Isolated From Surfaces on the International Space Station. Frontiers in Microbiology. 2019;10.

Sven abm. RNA Mango. Applied Biological Materials Inc[Internet]. [cited 2019 May 3]. Retrieved 2019 from: https://bit.ly/2ojoGcT

Tenover F. C., Baron E. J., Peterson L. R., Persing D. H. Laboratory Diagnosis of Clostridium difficile Infection: Can Molecular Amplification Methods Move Us Out of Uncertainty? Journal of Molecular Diagnostics. 2011;13(6):573-582.

Tong S. Y. C., Davis J. S., Eichenberger E., Holland T. L., Fowler, Jr. V. G. Staphylococcus aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. American Society for Microbiology. 2015;28(3):603-61.

Tortorello M.L., Stewart D. An Overview of Methods for Identification of E. coli O157:H7. In: Tunick M.H., Palumbo S.A., Fratamico P.M. (eds) New Techniques in the Analysis of Foods. Springer, Boston, MA. 1998.91-107 p.

Toze S. PCR and the detection of microbial pathogens in water and wastewater. Water Research. 1999;33(17):3545–56

Wang Z., Zheng P., Ji W., Fu Q., Wang H., Yan Y., Sun J. SLPW: A Virulent Bacteriophage Targeting Methicillin-Resistant Staphylococcus aureus In vitro and In vivo. Frontiers in Microbiology. 2016;7: 934.

Watson G. M., Kulkarni K., Brandt R., Del Borgo M. P., Aguilar M., Wilce J. A. Shortened Penetratin Cell-Penetrating Peptide is Insufficient for Cytosolic Delivery of a Grb7 Targeting Peptide. 2017; 2(2):670-677

Weisberger M. Live Science. Chinese Scientist Who Created Gene-Edited Babies Lied and Skirted Regulations, Officials Say. Retrieved 2019 January 22 from: https://bit.ly/2o7VTZ1

Zhang XH., Tee LY., Wang XG., Huang QS., Yang SH. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. Molecular Therapy Nucleic Acids. 2015;4(11):264.

Featured Image