Title

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The Exploration of CRISPR within Infectious and Genetic Disease Research, Diagnoses, and Treatments

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Abstract

Prokaryotes, such as bacteria, have evolved defense mechanisms that protect them from foreign bodies invading and harming them.[1] One of these mechanisms is the clustered regularly interspaced short palindromic repeats (CRISPR), alongside their accompanying CRISPR-associated (Cas) proteins.[1] This system functions as an immune response that protects prokaryotes from viruses (and other harmful bodies) by detecting foreign genetic material invading them and disabling their functionality and ability to spread.[2] Understanding the underlying mechanism of this immune response allowed scientists around the world to develop CRISPR and adapt it to various uses in gene editing, agriculture, and most recently, diagnosis of infectious and noninfectious diseases.[2] The discovery of CRISPR as a biomedical disease detection tool has revolutionized modern day medicine and its accessibility.

Infectious diseases are those that are caused by microorganisms and easily passed from one human to another. Currently, CRISPR--specifically the Cas13 protein system--is being utilized for disease detection and diagnosis through the detection and cleavage of specific ssRNA molecules. SHERLOCK, Specific High-sensitivity Enzymatic Reporter unLOCKing, is a specific breed of CRISPR Cas13a disease detection technology that has been deployed in West African countries to combat infectious disease such as Lassa Fever-- a disease caused by the zoonotic virus *Lassa mammarenavirus* that originates from the *Mastomys natalensis* rodent group.[5] SHERLOCK's ability to succeed in the detection and diagnosis of Lassa fever in West African countries that lack proper infrastructure relies on SHERLOCK's accessibility and efficiency. Cas13a's specificity and sensitivity in the detection of viral nucleic acids caters to the fact that Lassa Fever has high genetic diversity and requires highly sensitive tools to detect the disease.[8]

Moreover, genetic diseases are those caused by an error in the DNA of a person. The three main types include monogenic, complex, and chromosomal; the treatment and causes of the

diseases are different for each type.[17] There are still many obstacles when it comes to curing genetic diseases, yet scientists are using powerful tools such as CRISPR to help those who suffer from these diseases.[18] The CRISPR Cas9 enzyme is one powerful system that can be used to disrupt, delete, or insert genes to help edit any errors in the genome and treat genetic diseases.[23] It is comprised of the CRISPR-associated (Cas) enzyme and the guide RNA (gRNA), which work together to act as a scissor for the DNA.[19] Adrenoleukodystrophy (ALD) is an X-linked genetic disease that is primarily due to a mutation within the ABCD1 gene. Currently, the Cas9 system provides a disease model in order to study the pathogenesis of ALD and target (and repair) the mutation causing this genetic disease.

Introduction

In the following paper, we will be exploring the various implications of CRISPR on infectious and genetic diseases, specifically focusing on Lassa Fever and Adrenoleukodystrophy (ALD) respectively. Overall, we will detail why CRISPR is so beneficial in regards to these diseases and how it is being used as a tool. As of now, we know that various CRISPR Cas enzyme systems are used either as diagnostic tools or within gene mutation editing treatments. With CRISPR, diagnosing both infectious and genetic diseases has become more efficient, cheaper, and accessible. This is especially important in developing countries that lack the resources necessary for proper medical treatment, countries where diseases like Lassa Fever run rampant. Furthermore, CRISPR Cas systems provide disease models that help study the pathogenesis of diseases, as well as directly treat mutations. This is especially beneficial for genetic diseases like ALD. This review will further detail the connection between CRISPR, diagnostic tools, and treatments, as well as dive into two specific Cas enzyme systems: Cas9 and Cas13. In general, Cas9 is primarily used in correspondence with DNA, and Cas13 with RNA.

However, we will mainly detail how Cas9 is used to treat and create a disease model for ALD, while Cas13 is used to diagnose Lassa Fever.

Emergence of CRISPR Systems

Cas9

There are two main parts that are essential to the CRISPR Cas9 system: the CRISPR-associated (Cas) enzyme and the guide RNA (gRNA). The Cas9 is an endonuclease that acts as a pair of "molecular scissors" capable of cutting specific strands of DNA.[19] The gRNA guides the Cas enzyme in cutting the genome through two parts: the pre-designed RNA sequence (about 20 bases) that guides Cas9 to the right parts of the genome, as well as the longer RNA scaffold that ensures Cas9 cuts at the right point.[19]

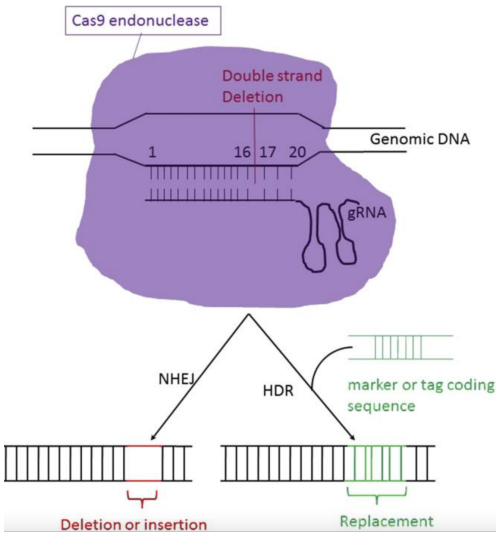


Figure 1. Diagram of how the gRNA guides the Cas9 system and the possibilities after.[20]

The pre-designated RNA sequences are designed to be complementary to the targeted DNA strand, and the gRNA will theoretically guide Cas9 to that sequence.[19] From here, there are three main options that the CRISPR-Cas9 system can take to edit the genes:

The first option is to disrupt the gene by making a single cut through both strands of the DNA using Cas9. This will change the DNA through a process called non-homologous end joining, which is a repair method in which the two cuts are glued back together. Essentially, this helps inactivate the targeted gene by adding or deleting base pairs. However, it is important to note that this method is more prone to errors.[23]

The second option is to delete the gene by using two guide RNAs that target and delete separate fragments of DNA. This is also through non-homologous end joining, which causes the separate ends to unite and delete the intervening sequence.[23]

The third and final option is to correct or insert a gene by adding a DNA template with the CRISPR system. Through a process called homology-directed repair, a sequence of nucleotides is inserted into the gap in the sequence of nucleotides instead of gluing it back together.[23]

Cas13

When Cas9 was discovered, it revolutionized biomedical research and medicine and now remains one of the most-known Cas proteins. However, its main use is specific to gene editing. The discovery of Cas13 expanded the use of CRISPR to include disease detection and diagnosis. The system is composed of a single-component enzyme that depends on a specific RNA sequence as a guide.[3] Once Cas13 becomes RNA guided--whether it's naturally occurring in bacteria or engineered in a lab--it is then able to detect and bind to a specific single-stranded

RNA (ssRNA). Cas13 is characterized by its ability to detect this ssRNA, while simultaneously collaterally cleaving RNA that is not targeted.

Cas13 is used to engineer and create easily used, highly accessible, and cheap diagnostic tools in order to detect infectious diseases caused by viral infections--specifically by targeting their RNA.[3] In 2017, Gootenberg introduced one of these tools: the specific high sensitivity enzymatic reporter unlocking (SHERLOCK).[2] SHERLOCK is a highly sensitive diagnostic tool that is used to detect DNA/RNA strands in urine, saliva, serum, plasma, and blood.[8] The ability to detect DNA/RNA with high accuracy and sensitivity with virtually no need for expensive materials and labs is important for clinical diagnosis of infectious diseases and stopping the spread of them.[8]

Introduction to Genetic Diseases

Today, there are around 300 million people worldwide who suffer from at least one of the 6000 known genetic diseases.[16] These disorders happen when there is a change or mutation within the DNA of a person. In a human, the genome is comprised of 46 chromosomes--22 pairs of autosomal chromosomes and 2 sex chromosomes--which all contain almost 3 billion base pairs of DNA that are responsible for over 30,000 protein-coding genes.[16] These protein-coding regions make up less than 5% of the genome. Scientists are still unclear about the precise function of the remaining 95% of DNA.[15]

Genetic diseases usually fall within the 3 main categories of monogenic, complex, and chromosomal disorder:[17]

Monogenic disorders (single-gene inheritance) result when there is a change or mutation in one particular gene. These are inherited in different ways (recessive, dominant and X-linked), but often have more predictable inheritance patterns because they are easier to track through familial lines.[17] Examples of this type include Cystic fibrosis, Sickle-cell anemia, Marfan syndrome, Duchenne muscular dystrophy, and Huntington's disease.[17]

Complex disorders (multifactorial inheritance) are caused by a combination of genetics and one's environment or lifestyle. Factors that affect the chances of these diseases include, but are not limited to, smoking, drinking alcohol, unhealthy diets, exposure to high air pollution, and lack of sleep. Examples of this type include asthma, heart disease, diabetes, certain cancers, schizophrenia, and Alzheimer's disease.[17]

Chromosomal disorders occur when one has an additional chromosome, lacks a chromosome, or has a chromosome that has structural abnormality. Although these can be hereditary, chromosomal anomalies usually occur due to problems with cell division within the egg or sperm (gametes).[17] Examples of this type include Down syndrome, Klinefelter syndrom, Patau syndrome, Edwards syndrome, and Turner syndrome.[17]

A huge obstacle in curing genetic diseases is that there are many other factors that play a role in the development of these disorders. For example, with multifactorial inheritances, no single gene has the power to dictate whether the person will obtain the disorder.[18] Most often, it is due to the fact that more than one mutation occurs, or multiple genes make subtle contributions.[18] Because of this, gene therapy has mainly been targeting monogenic diseases, in

which the most commonly used Cas nuclease is SpCas9 that is derived from Streptococcus pyogenes and was programmed for genome editing in mammalian cells.[18] However, there have been many additional discoveries that have given rise to other Cas9 proteins, such as those from Staphylococcus aureus (SaCas9) and Neisseria meningitidis (Nme2Cas9).[18] With these different types of proteins, the possibility and applications of these CRISPR systems have increased and enabled more advanced gene editing.[18]

A common method that scientists currently use to understand genetic mechanisms and develop solutions against them, is the use of model organisms (mice being the most prevalent). This practice can be used to generate disease models in large animals.[18] For example, when researchers were studying Parkinson's disease and attempting to understand it better, they used a monkey model. With that, scientists introduced a PINK1 (a type of gene) deletion and revealed that a developing primate brain needs a functional PINK1.[18]

When studying something as powerful as CRISPR, the limitations also need to be acknowledged, along with the potential dangers. One of the biggest trade-offs includes the off-target effects of using CRISPR, as RNA and DNA editing can always go wrong.[18] These effects are very dangerous on the genomic level because they can cause large deletions and genomic rearrangements.[21] These can then lead to lethal genetic mutations, which include the loss of gene function and potential cancerous cells.[21] Scientists hope that by using single guide RNA selection and optimization, the chances of this will be reduced.[18] Another big concern is the immune response--essentially when a person's body fights against the gene therapy--that is stimulated by using CRISPR. In a recent study, the effects of anti-Cas9 responses were

demonstrated using a sample of 34 human blood cells.[18] 79% of the samples contained antibodies against SaCas9 and 65% of the samples contained antibodies against SpCas9.[18] In order to fully use the potential of CRISPR to tackle genetic disease, this effect will have to be addressed in clinical applications.[18]

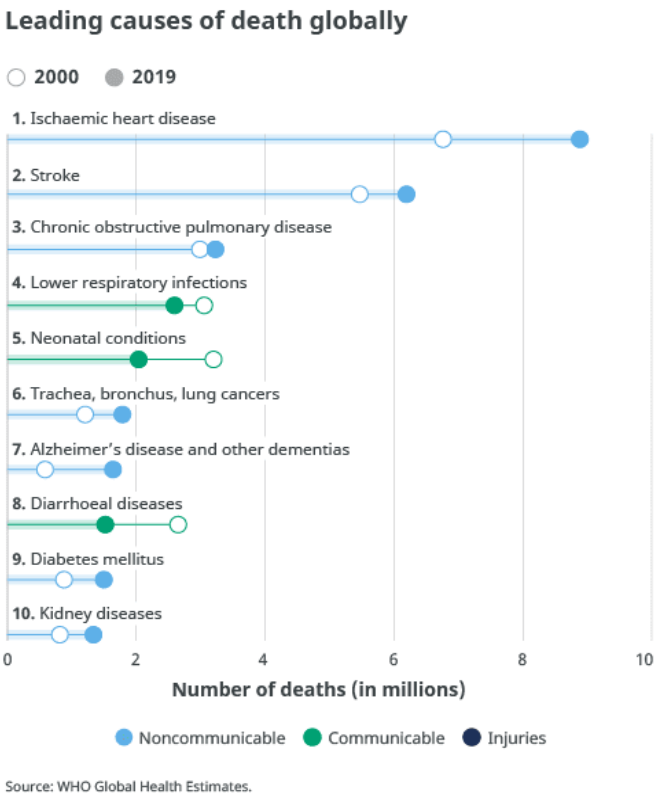
Introduction to Infectious Diseases

Rapid and accurate diagnosis is crucial to the detection and treatment of any disease. This is specifically relevant to infectious diseases since they kill millions of people globally and require a fast response to limit their spread of infection.[4] However, the widely-used diagnostic tools currently introduce various limitations, ranging from pricing to assembly. Thus, there's a need for user-friendly, cheap, and accurate diagnostic tools that can be widely accessible and overcome the previously mentioned limitations. One such tool is CRISPR-Cas13.[3] To understand how the Cas13 system can be used in the detection of infectious diseases, the pathogen-host interaction must be thoroughly understood.

Infectious diseases are usually caused by microorganisms which include fungi, parasites, and viruses. The infection occurs when these harmful microorganisms are passed from one person to another. Moreover, infectious diseases are not only spread as a result of human-to-human contact: they can also be passed from an animal to humans. The danger of infectious diseases lies in the fast spread of these microorganisms, as well as their potential fatality to anyone exposed to them.[2]

In 2019, the World Health Organization (WHO) classified 3 out of the top 10 causes of death worldwide as communicable diseases, which include infectious and parasitic diseases.[4]

These diseases are as follows: lower respiratory infections, neonatal conditions, and diarrhoeal diseases. The WHO report also states that low-income countries are at a higher risk of exposure to these diseases. This threat in fact expands beyond the previously mentioned three infectious diseases to include malaria, tuberculosis, and HIV/AIDS.[4] This indicates that the detection, prevention, and control of infectious diseases depend heavily on countries' accessibility to resources and tools, or lack thereof. Thus, addressing and treating infectious diseases requires finding solutions and creating tools that are widely accessible beyond the barriers of wealth. Diagnostic tools, if transformed, could be one of these solutions that surpasses such barriers.



The focus of this review is *Lassa mammarenavirus* (LASV), the virus that causes Lassa Fever (Figure 2) [5] Lassa fever is a zoonotic virus characterized by its severe fevers, muscle aches, sore throat, nausea, vomiting, and chest/abdominal pain. Its prevalence in West African countries such as Sierra Leone, Guinea, Liberia, and Nigeria has caused an upwards of 300,000 to 500,000 cases with 500 deaths on a yearly basis.[5]

Viruses have different types and shapes, but they all have two major components: nucleic acid (DNA or RNA) and a capsid (a protein coat).[6] For LASV, the nucleic acid is a single-stranded RNA (ssRNA) virus.[5] Viruses in general rely on the cells of their host in order to replicate, regardless of their type of nucleic acid. *Figure 2* illustrates how viruses generally replicate using their hosts' cells and cellular machinery.[6] First, the virus inserts its nucleic acid into the cytoplasm of the cell. Once it is inside, the nucleic acid is replicated and also translated to synthesize the viral proteins. The replicated nucleic acids are then joined by the newly synthesized proteins to assemble new copies of the virus which are now ready to exit the cell and attack others.[6]

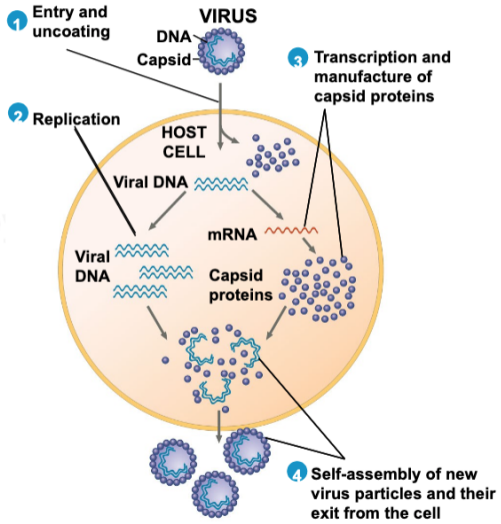


Figure 2. Replication and assembly of viruses using the host's cells.[6]

CRISPR and Adrenoleukodystrophy

X-Linked Adrenoleukodystrophy (ALD) is a genetic disease caused by a mutation in the ABCD1 gene. In spite of there being no relation between the genotype and the severity of the phenotype, the common feature shared amongst ALD patients is the ABCD1 mutation. This gene

is responsible for encoding the ABCD1 protein, which when mutated results in a protein defect that prevents the body from breaking down long-chain fatty acids (VLCFAs).[13] This causes VLCFAs to build up in the adrenal gland and nervous system, as well as contributes to the three main phenotypes that can be observed in affected males: inflammatory cerebral demyelination, progressive myelopathy, and adrenal insufficiency.[13] X-linked Adrenoleukodystrophy exists in two main clinical forms: a childhood (cALD) and adult form (AMN). cALD is the cerebral inflammatory version and is far more severe than AMN (adrenomyeloneuropathy) because it progresses faster.[14] The severity of the disease is associated with the disruption of the myelin sheath which is responsible for the insulation of nerve cells in the brain. X-linked Adrenoleukodystrophy is a quite rare disease with an overall frequency of 1:17000 and provides more severe complications for men than women affected.[13] [Since ALD is X-linked, it is most likely to affect men more than women. Men have only one X chromosome, while women have two. Thus, women can have one X chromosome with the mutation and the other without--men don't have this luxury. Overall, it is very rare for a woman to have ALD and more likely that she is merely a carrier.] In terms of treatment, hematopoietic stem cell transplantation is the current effective therapy as it aids in ceasing demyelination. However, not many affected people are able to receive hematopoietic stem cell transplantation as it is only meant for targeting early cerebral inflammation.[13] This is where the door opens for the CRISPR Cas9 system.

CRISPR Cas9 is primarily used to correct ALD patient-derived iPSCs. Normally, correcting the overproduction of VLCFAs has been attempted by targeting pathways alternative to ABCD1 (such as ABCD2 and ABCD3), or by mediating inflammation and oxidative stress. At a clinical level these methods seem to be less effective outside of in vitro/vivo.[13] The CRISPR

Cas9 system can not only provide a disease model to study the pathogenesis of a disease, but it can correct the mutation causing ALD using homology-directed repair.[13]

Furthemore, CRISPR Cas9 has played a further role in being able to dissect the pathogenesis of X-linked Adrenoleukodystrophy. As mentioned, the ABCD1 gene is critical in the pathogenesis of ALD. The ABCD2 gene is homologous to the ABCD1 gene as it encodes a peroxisomal half-ABC transporter and is also important in the disease model.[14] Microglia, the surrounding immune cells which help maintain the central nervous system, have been found to play a significant role in both clinical forms of ALD. This role involves their activation due to environmental signal responses, leading to certain metabolic changes as well as to the accumulation of VLCFAs. Additionally, the presence of microglia greatly increases in lesioned areas that result from X-ALD-related inflammation. In 1990, a respectable alternative to microglial cells, the BV-2 immortalized cell line, was established.[14] In a study, the BV-2 cells were used to create an Acox1 deficient microglial cell line by means of CRISPR Cas9 gene editing. The purpose of this newly derived cell line is to help with the analysis of peroxisomal dysfunctions as the Acox1 gene has the job of encoding the rate-limiting enzyme in the peroxisomal-beta oxidation pathway.[14] As the BV-2 cells express both the ABCD1 and ABCD2 proteins, new BV-2-derived cell lines with single/double mutations in the Abcd1 and/or Abcd2 genes can be created and studied. In the respective study, 3 types of mutant BV-2 cell clones were created: Abcd1-, Abcd2-, and Abcd1/Abcd2-deficient. [14] CRISPR Cas9 aided in generating the clones by mediating a double strand DNA break. With the mutant cell clones as independent variable models, the physiopathology of X-ALD can be better understood and studied. For example, one of the tests was on fatty acid levels in response to the absence of the peroxisomal

ABC transporters.[14] The results showed that the Abcd1/Abcd2-deficient cells seemed to be most reminiscent of X-ALD in terms of VLCFAs when compared to the single mutant Abcd1- and Abcd2- cells. Additionally, the visual structural areas in the double mutant cells were very similar to the demyelinated areas in ALD patients. All in all, CRISPR Cas9 made the generation of the mutants possible, and in doing so opened a whole new avenue for the way ALD can be studied through a disease model.[14]

CRISPR and Lassa Fever

LASV originates from the *Mastomys natalensis* rodent group.[5] However, these rodents tend to not show any clinical symptoms of the virus and are lifetime carriers of it.[5] Infected rodents excrete and spread the virus through saliva, urine, respiratory excretion, and blood.[5] Humans then can come in contact with the virus through their respiratory tract or faecal-oral system. The spread of LASV between humans occurs when a person is exposed to the blood, semen, saliva, feces, or air of an infected person, therefore posing a tremendous risk to the population because of the ease of transmission. In addition, an infected person can secrete the virus for up to thirty days, further increasing the ease of transmission. Unlike some zoonotic viruses, the spread of LASV can occur during any part of the year, allowing for an even higher transmission rate.[5]

Due to the prominence of LASV in West African countries and its ability to spread rapidly, accessibility to quick testing is much needed. The lack of proper infrastructure and resources in these countries has made it widely difficult to control and halt the spread of the virus. The inability of these countries to lessen the spread of LASV is attributed to the lack of accurate and cost effective diagnostic tools, thus resulting in the spread of the virus in countries

outside of the endemic regions. SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing)--a CRISPR-Cas13 diagnostic tool that is used to detect specific DNA/RNA in a given concentration--is known for being one of the most specific and effective diagnostic tools on the market. It is currently being investigated to be used on LASV and has the potential to revolutionize diagnostic tools. SHERLOCK is highly accessible, accurate, fast, and can be easily used and distributed--in fact, this "user-friendly" diagnostic tool can potentially reduce the death rate for Lassa fever by 60%.[5]

In order to slow the spread of Lassa fever, quick and effective diagnosis is required. However, the current method of polymerase chain reaction (PCR) based diagnostic tools utilized to detect Lassa fever lack the ability to effectively diagnose patients in areas of poor infrastructure. Although PCR-based diagnostic tools are adaptable to mutating variants of diseases, the tests require laboratories for results to be processed and are not cost effective because expensive reagents are used.[11] Antigen testing is another method used to diagnose Lassa fever, yet it is also ineffective due to its inability to detect the virus at its early stages.[7] With its high genetic diversity, LASV provides a significant challenge in the creation of an adaptable diagnostic tool that would detect all strains of the virus at every stage of its development.

CRISPR gene editing technology has introduced the SHERLOCK platform as a rapid diagnostic tool using Cas13 proteins. What sets SHERLOCK apart from PCR and antigen testing is its high sensitivity and specificity, which both specifically target the high genetic diversity of LASV. In addition, SHERLOCK is cost effective--about $0.60 per unit--and it does not require sophisticated, high end laboratories, making it the ideal diagnostic tool for areas that lack the proper resources.[7] [In comparison, PCR testing ranges from $15-25[11] and antigen testing from $5

per test. Furthermore, both diagnostic tools require sophisticated laboratory resources.[12]] Most importantly, SHERLOCK is very sensitive with its detection of molecules, in fact it can detect a single molecule in a 1-µl volume of RNA and DNA.[7] This ultrasensitivity is not lessened over prolonged storage periods, making SHERLOCK an even more desirable tool for long term usage.

Cas13a allows SHERLOCK to be developed as a diagnostic tool, rather than a gene editing tool because Cas13a proteins bind and cleave RNA substrate, instead of DNA substrates.[8] After Cas13a cleaves the target RNA substrate, the substrate switches to an active enzyme state.[8] Cas13a proteins then continue to bind and cleave onto surrounding RNA substrates. This is called collateral cleavage. Since Cas13a has the ability to detect a specific target RNA instead of a DNA substrate in order to become activated through collateral cleaving, it can then be utilized as a highly efficient diagnostic tool in infectious disease detection.[8]

In infectious diseases such as LASV, the viral nucleic acids require highly sensitive and specific diagnostic tools to detect their presence. The RNA-targeting proteins of Cas13 are used by SHERLOCK to detect these viral nucleic acids .[8] More specifically, SHERLOCK is able to pair recombinase polymerase amplification (RPA) with Cas13a detection, allowing for Cas13a to be paired with the specific targeted sequence of the nucleic acids and signal amplification.[9] Because RPA cannot distinguish between single-base-pair distinctions in the target sequence, it must be paired with Cas13a due to Cas13a's specificity and sensitivity in detection of viral nucleic acids.[8] Amplification and Cas13a detection are performed isothermally, meaning a relatively low amount of energy is utilized. In addition, this allows for pipettes to be utilized

during testing, making point-of-care detection ideal. It is important to note that the isothermal property of SHERLOCK means a single temperature is needed, thus excessive instrumentation and tools are not required to perform the testing or to retrieve results, making SHERLOCK more accessible and cost effective.[8]

While SHERLOCK is an accurate and effective diagnostic tool on its own, it can be used in combination with other diagnostic tools to strengthen its ability to be used as a point-of-care tool and eliminate the need for excess instrumentation. As a result, this makes SHERLOCK suitable for areas that lack proper infrastructure. HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases) is a diagnostic tool that is used in conjunction with SHERLOCK to ensure the safe and efficient testing of infectious diseases.[9] HUDSON eliminates the need for column or bead based nucleic acid extraction. Therefore, it can test for diseases through bodily fluids, minimizing the amount of infectious materials/RNA that is needed to be tested.[9] This also eliminates the risk of infection for healthcare workers.

With the help of Cas13, diagnostic tools for detecting LASV have been immensely improved and made more cost and resource efficient. As a result, this allows for countries lacking money and special equipment to detect a virus that is traveling so freely through its people.

Conclusion

Both CRISPR Cas9 and Cas13 systems are important, budding tools within the scientific community. Each has its own specificity that is currently being studied, improved, and tested. Cas9 has mainly been employed in genetic disease treatment, diagnoses, and research. On the

other hand, Cas13 has primarily been utilized in regards to infectious diseases and diagnosing them.

Lassa Fever is a virus that stems from the *Lassa mammarenavirus* (LASV).[5] With the help of Cas13, the diagnoses and detection of LASV has been vastly improved. Furthermore, perhaps more importantly, CRISPR has made a more resource and money efficient diagnostic tool that vastly benefits low-income countries where Lassa Fever is most prevalent. These countries do not have the means necessary to detect LASV using more expensive, resource-heavy tools. Cas13 is one way to combat this.

As for the genetic disease aspect of this paper, Cas9 and Adrenoleukodystrophy (ALD) were the primary focuses. This CRISPR Cas9 system provides a disease model that can help study the pathogenesis of ALD and repair the mutation (in the ABCD1 gene) that causes ALD using homology-directed repair.[13] This paper mainly focused on Cas9 as a tool to study ALD (with disease models) using the ABCD1 gene and its homologous gene, ABCD2.

CRISPR is a research, diagnostic, and treatment tool that has immense benefits for mankind. It is still being studied, with new advances being discovered every day within genetic and infectious diseases like Adrenoleukodystrophy and Lassa Fever. There must be more experiments done on the immune response of CRISPR, as well as studies on its accessibility and affordability when compared to other tools. As we learn more about CRISPR, we get one step closer to better treatments and diagnostic tools.

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