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Review article

REVIEW: CRISPR-Cas Methodologies in the Detection and Treatment of Infectious Illnesses

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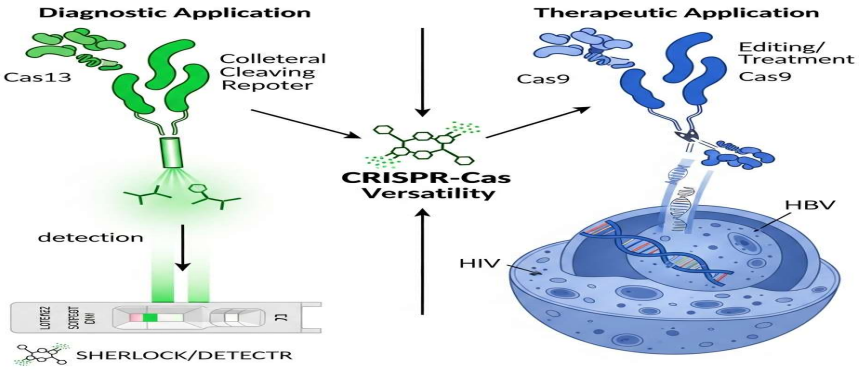
Graphical Abstract



Abstract: The identification of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their corresponding Cas proteins, originally a bacterial defense mechanism, has initiated a transformative phase in genetic engineering with significant ramifications for clinical treatment. This review examines the swift advancement and many uses of CRISPR-Cas techniques in the identification and management of infectious diseases, emphasizing their capacity to revolutionize contemporary clinical practice. In diagnostics, systems such as SHERLOCK (for RNA) and DETECTR (for DNA) employ the collateral cleavage activity of Cas nucleases (e.g., Cas12 and Cas13) to enable ultra-sensitive, isothermal, and quick detection of viral and bacterial nucleic acids, including SARS-CoV-2. These field-deployable devices, in conjunction with Next Generation Sequencing augmentation methodologies such as DASH and FLASH for low-frequency targets, signify a substantial advancement beyond conventional diagnostic techniques. Therapeutically CRISPR-Cas provides a means to achieve a functional cure for chronic and latent viral infections by accurately targeting and modifying integrated viral genomes that are typically unreachable by standard antivirals. Promising pre-clinical research has shown the eradication or inactivation of viruses such as Human Immunodeficiency Virus-1 (HIV-1) and Hepatitis B Virus (HBV) through tactics like multiplexed guided RNAs and less-mutagenic base editors. Although the remarkable promise is evident, issues of delivery efficiency, off target editing, and host immunity must be resolved as these technologies go into preliminary clinical trials. Ultimately,

CRISPR-Cas techniques represent a disruptive force, offering accurate, diverse, and readily changeable tools that are set to become essential in the clinical management of infectious diseases.

Keywords: CRISPR, Cas 9, Cas12, Cas13, infectious diseases

INTRODUCTION

CRISPR Discovery

Spanish microbiologist Dr. Franciso Mojica was the first to discover "Clustered regularly interspaced palindromic repeats" (CRISPR) in the archaeal species Haloferax mediterranei. These repeats are composed of palindromic sequences of 30bp separated by spacer sequences of 36bp (Chen et al., 2020). At first, there was no interest in the discovery beyond microbiology; however, the identification of analogous palindromic repeats in various bacterial species, including Mycobacterium tuberculosis, Escherichia coli, and Clostridium difficile, indicated that CRISPR repeats could fulfill a significant preserved function. "CRISPR-associated proteins", or Cas proteins, are protein-coding genes that perform nucleic acid unwinding and cleavage, were also identified near the CRISPR sequences (Koonin et al., 2017). The role of repeats of CRISPR were ultimately clarified when Dr. Mojica made a noteworthy discovery in 2003. Mojica found associations between specific sequences of CRISPR and viruses that infect bacteria called bacteriophages, during the search for homologies with other known sequences. Mojica proposed that given CRISPR sequences and their potential corresponding Cas proteins may operate as a protection against microbe's mechanism, retaining bacteriophage "memories" to combat future infections (Chen, 2020; Mojica, 2005). CRISPR's mechanism functionality in bacteria is notably straightforward (Figure. 1). When a bacteriophage or plasmid invades, Cas proteins break up the foreign DNA segments and incorporate them establishing a "memory" of the sequence within the CRISPR locus (Butiuc-Keul et al., 2022). To create an RNA transcript that includes every CRISPR repeat and its corresponding sequence, the CRISPR locus is continually transcribed by the bacterium. Pre-crispr-ribonucleic acid (pre-crRNA) is the name given to this transcript. CRIPSR ribonucleic acids (crRNAs), which include a single spacer and a single CRISPR repeat, are produced when a Cas protein cleaves the pre-crRNA (Faure et., 2019). The crRNA segments are released into the cytoplasm to eliminate invading pathogens, where they identify and bind to complementary DNA or RNA sequences. Upon the formation of a match, a cleavage complex is constructed comprising the crRNA, its target sequence, Cas nucleases, and a short RNA known as trans-activating CRISPR RNA (tracr RNA). The protospacer adjacent motif (PAM), a location next to the crRNA target sequence, is where this complex cleaves the target. Numerous bacterial species' Cas proteins aid in the cleavage of nucleic acids through distinct mechanisms. Certain Cas proteins, such as Cas9, operate alone, whereas others assemble into cleavage complexes comprising numerous Cas proteins. Regardless of the separation process, the CRISPR technology functions as a microbiological protection mechanism, specifically focusing on the RNA or DNA of intruding entities for breakdown and removal.

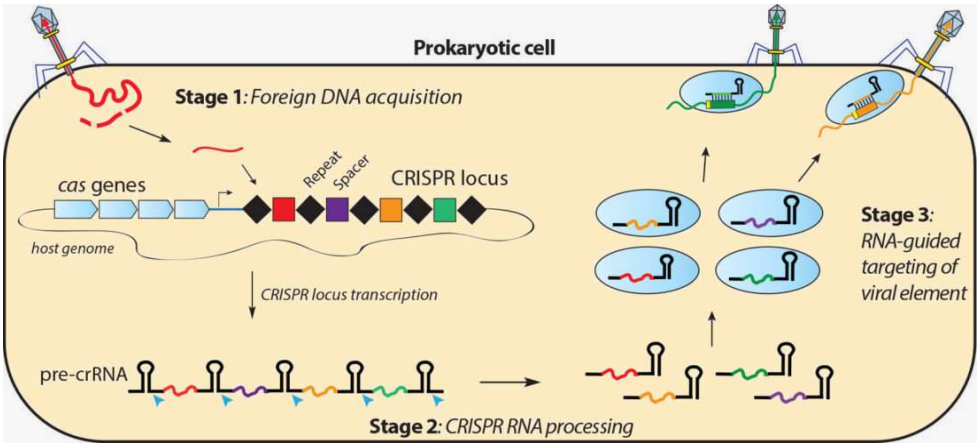


Figure 1: The CRISPR mechanism in prokaryotes. Foreign viral DNA is sequestered by Cas proteins and integrated as a novel spacer into the CRISPR locus, establishing a genetic record of infection. The CRISPR array undergoes transcription and is subsequently processed into distinct crRNAs, each containing a specific spacer sequence. Mature crRNAs direct Cas effector complexes to corresponding viral DNA, facilitating accurate cleavage and elimination of the intruder.

Gene Editing through CRISPR

In 2013, the initial report on human gene editing utilizing CRISPR was published by Dr. Feng Zhang from the Massachusetts Institute of Technology (Cong et al., 2013). His study was based on the innovative discoveries of Jennifer Doudna and Emmanuelle Charpentier, who had previously determined the essential components needed for cleavage mediated by CRISPR (Jinek et al., 2012). Recognizing that CRISPR procedure could make precisely intended changes inside human genes, Zhang and his colleagues devised a procedure to produce tracrRNA, crRNAs and the Cas 9 cleavage protein inside human cells (Cong et al., 2013). Repairing broken DNA at the target locus was one of Zhang and his team's major challenges. Without intervention, the repair of served DNA ends transpires through NHEJ (Figure. 2). This procedure is susceptible to errors, frequently causing indels to be added or removed, hence causing unanticipated alterations in the genetic code. Consequently, the intended gene could remain inactivated or be restored upon repair. Zhang and his colleagues hypothesized that the served DNA end ends might be restored using homology-directed repair (HDR), in a template-specific way, by introducing a homology repair template into the cells in conjunction with the CRISPR system. By employing this technique, Zhang and his group were able to cause the EMX1 homeobox gene in human cells to cleave and repair via HDR (Cong et al., 2013). When compared to alternative gene-editing methods, the main benefits of CRISPR gene editing's specificity and precision, even for extremely tiny regions. This results from both accuracy of Cas separation at the target site and the strong complementarity between the crRNAs and their targets (Shmakov, 2017; Doetschman, 2017). Furthermore, the simplicity of CRISPR is a benefit, as it only needs a tracrRNA, the Cas9 nuclease protein (from Streptococcus pyogenes), and a crRNA guide sequence to function (Doetschman, 2017; Liu, 2015). You can get more features by employing CRISPR-associated nuclease from other microorganisms. For example, Leptotrichia shahii's Cas13 protein is an RNA-specific nuclease (Shmakov et al., 2017). Proteins called Cas have been altered to carry out a variety of enzymatic processes, including base editing and DNA methylation. These improvements have significantly enhanced the nucleic acid modification capabilities of CRISPR-oriented systems (Ranzau, 2018; Kang, 2019). CRISPR technology presents extensive possibilities across various biological domains, including agricultural production and environmental science. In the field of medicine, CRISPR-oriented therapies for genetic disorders and tumors are being developed, with several now in clinical trials (Stadtmauer et al., 2020). CRISPR technology's application in the treatment of infectious diseases is among its most exciting uses. Cost-effective and trustworthy CRISPR diagnostics for bacteria and viruses are being developed, and CRISPR-oriented treatments are being developed to cure a variety of difficult infections, such as HBV, MRSA, and HIV-1. CRISPR systems are easily adaptable to target new infections or resistance pathways, in contrast to conventional diagnostic and treatment techniques. Because of this, they offer a potent potential weapon against newly developing viruses, where development speed is crucial. Both "off-target" and "on-target" consequences are hazards associated with CRISPR-based therapies, and as CRISPR technologies advance from the lab to the patient's bedside, these concerns will need to be addressed.

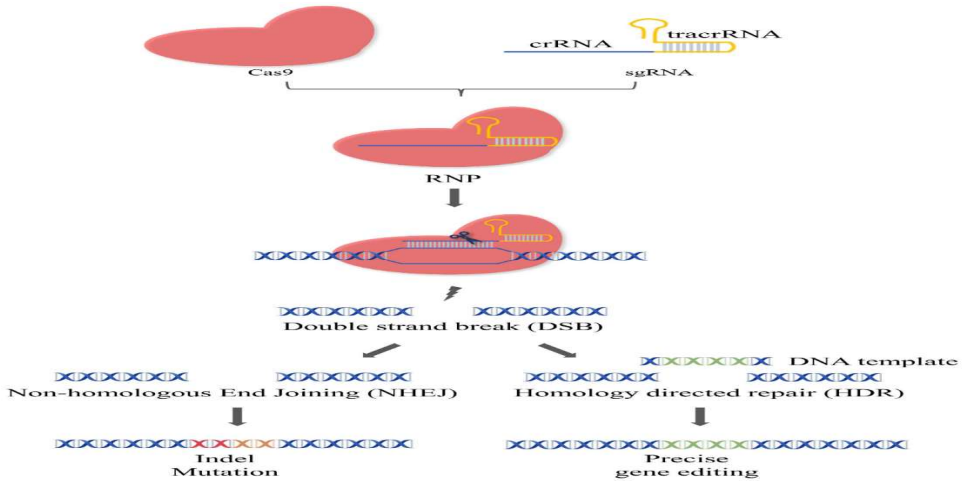


Figure 2: Mechanism of CRISPR Cas 9. A specific double-strand breaks in DNA is introduced by the CRISPR-Cas9 ribonucleoprotein complex, which is made up of CAS9 and one guide RNA. Either homology-directed repair employing a donor template for accurate gene alteration or error-prone non-homologous end joining, which results in indels, is used to fix the break.

Diagnostics based on CRISPR

Systems based on CRISPR offer increased pace and precision in the identification of bacterial and viral nucleic acids (Yuen, 2018; Kim, 2021). DETECTR (Yang et al., 2015) and SHERLOCK (Niu et al., 2017) are two similar diagnostic methods that have been developed to identify DNA and RNA sequences, respectively. First, recombinase polymerase amplification (RPA) is used to amplify bacterial or viral genomes from clinical samples. After that, the resultant DNA is combined with a CRISPR/Cas system, which recognizes and cuts the desired sequence. This reaction's Cas endonuclease is designed to exhibit indiscriminate cleavage activity only after attaching itself to its target and becoming active. For RNA (DETECTR), the enzyme utilized is Cas12a (Yang, 2018; Smith, 2020), whereas for DNA (SHERLOCK), it is Cas13a (Niu, 2017; Zhao, 2022). Cas12a and Cas13a begin the indiscriminate cleavage of additional DNAs or RNAs in the solution after cleaving their target regions. Consequently, upon the introduction of a quenched reported sequence, the sequence will be cleaved by Cas12a or Cas13a, producing a signal that is fluorescent. Nevertheless, the reporter sequence will stay quenched and Cas12a and Cas13a will not be activated if the target sequence is absent from the solution. Because DETECTR and SHERLOCK are extremely sensitive, they can detect viruses with just one copy. Additionally, the enzymatic reactions don't require costly heat cyclers and operate at 37 ºC. Additionally, they are quick. They only take one to two hours to complete. Because of these factors, there is a lot of interest in employing DETECTR and SHERLOCK as viral diagnosis instruments that may be used in viruses including dengue, Ebola and Zika (Kim, 2021; Myhrvold, 2018). Recent reports indicate that SARS-Cov-2 detection utilizing DETECTR achieved a turnaround time of less than forty-five minutes, exhibiting 100% negative and 95% positive predictive agreement with PCR techniques (Figure 3) (Eftekhari et al., 2021). By increasing the sensitivity of NGS, CRISPR techniques can also be used to make it easier to identify low-frequency sequences in medical specimens, including genes that resist antibiotics. CRISPR/Cas9 is used in the pre-processing step of the DASH approach to cleave high frequency targets, including human mitochondrial ribosomal RNA genes, that reduce NGS's sensitivity. The relevant pathogenic sequences can thus be easily amplified when these high-frequency targets have been removed. Using this method, fungal and parasite sequences from cerebral fluid samples were amplified with significantly higher sensitivity (Hardigan et al., 2019). Alkaline phosphatase is used in FLASH, and alternate CRISPR-based techniques, to obstruct each sequence in the sample. The target sequence is then partially digested using a CRISPR/Cas system, connected to universal sequencing adaptors, and enhanced with minimal amplification of additional sample sequences. Tracheal aspirates from patients with Staphylococcus aureus pneuomonia were examined using FLASH-NGS to find genes that resist antibiotics. Compared to NGS alone, the method produced a 5000-fold enrichment of the target genes. Additionally, it was effective in identifying genes resistant to malaria from dried samples of blood spots (Nafian et al., 2023). Once more, compared to NGS alone, FLASH-NGS had far higher detection sensitivity. Clinical laboratories are likely to quickly adopt these remarkable approaches. They are not only extremely sensitive but also quick and affordable, which makes them an alluring combination (Ai et al., 2019). Furthermore, there aren't any serious patient dangers that need to be considered, in contrast to CRISPR therapies.

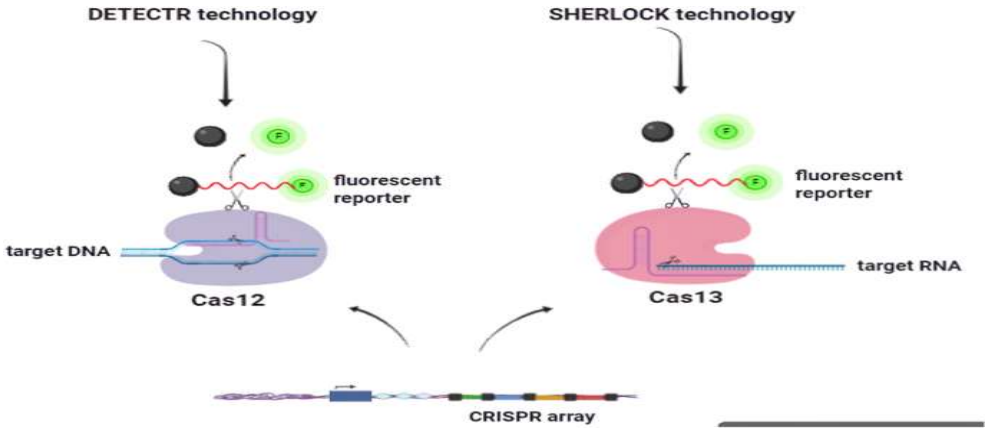


Figure 3: DETECTR uses Cas12 to identify and cleave target DNA, initiating collateral cleavage of a fluorescent reporter, whereas SHERLOCK utilizes Cas13 to detect target RNA and similarly induce fluorescence via collateral RNA cleavage. Both technologies utilize CRISPR-derived guide RNAs from the CRISPR array to direct nuclease specificity for precise nucleic acid identification.

CRISPR-Based Therapeutics for the Management of Chronic and Acute Viral Infections

Viruses are creatures made of encapsulated viral DNA or RNA that enter living cells and take control of their internal processes to replicate themselves. They are clear candidates for CRISPR-based gene editing treatments due to their intracellular location and need on cellular proteins for survival and replication. There are three different types of viral infections: latent, chronic, and lytic. Actively, the virus multiplying and creating virions, or viral progeny during lytic infection. Following their packaging into capsids, or protein coats, the viral genomes are liberated by cell lysis, which results in the host cell's death (Traylen et al., 2011). Additionally, a few viruses may result in latent infection which is a condition in which the virus goes into a dormant state and state there for years. Latent infections are caused by several significant human diseases, such as Epstein-Barr virus (EBV), parvovirus, cytomegalovirus, human herpesvirus 6 (HIV-6), hepatitis B virus (HBV), polyomavirus, and Herpes Simplex 1 (HSV-1) (Traylen, 2011; Lieberman, 2016). Lastly, persistent low-level viral replication that results in long-term organ damage is known as chronic infection. Several significant diseases, such as HIV-1 and HBV, are characterized by chronic infection (Virgin et al., 2009). About 40 million individuals have HIV-1 infection, and 250 million have chronic HBV infection (Liu et al., 2023), these two illnesses generate significant morbidity and mortality. The viral genome is perpetually preserved within the host cell in both latent and chronic viral infections, either as free-floating viral micro chromosomes or incorporated into the host genome. It is extremely difficult to treat a virus once it has established itself inside the host cell. Nevertheless, viral genomes within the host cell can be targeted by CRISPR-based technologies, which prevent transcription and replication. Therefore, a potential treatment for these challenging chronic infections in CRISPR gene editing. Despite the idea's natural appeal, there are practical obstacles that must be addressed. Delivering the CRISPR system to the target cells is the first step. Technology also needs to specifically target viral genomes that are constantly evolving; this is especially difficult with HIV-1 due to its substantial genetic heterogeneity even within a single host and fast pace of mutation (Hadj Hassine et al., 2022). Third, a strategy needs to be in place to prevent different viral reservoirs within the body from infecting the previously "cured" cells. Lastly, the technology must not cause unforeseen consequences at "off target" locations within the host genome. The development of CRISPR-based treatments for persistent viral infections has advanced significantly despite these obstacles. A CRISPR/Cas9 technology was employed in a study to reduce HBV in a mouse model and a human hepatocyte cell line (Ramanan et al., 2015). It is encouraging that after four weeks of continuous CRISPR/Cas9 expression in the mouse model, no off-target cleavage events were observed. A separate study utilized a modified Cas9 protein for "base editing" to target HBV (Figure 4) (Yang et al., 2020). The Cas9 protein for base editing created a nonsense codon that stopped the translation of viral proteins by inducing nucleotide alteration rather than cleaving the DNA at the target location. This base editing technique minimizes the possibility of chromosomal changes at the intended location (which may result in cancer) and the chance of off-target effects by preventing the formation of double-strand breaks.

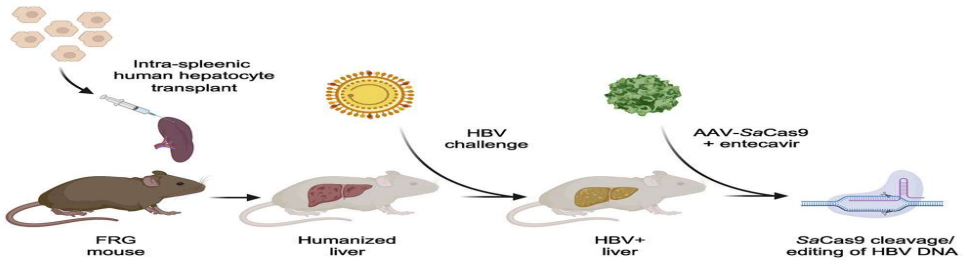


Figure 4: FRG mice are humanized through intra-splenic transplantation of human hepatocytes and subsequently challenged exposed to HBV to induce infection. The HBV-positive mice are subsequently administered AAV-delivered SaCas9 and entecavir, facilitating targeted cleavage/editing of HBV DNA in humanized liver tissue.

HIV-1 is a multifaceted and evasive virus, and its capacity for mutation enables it to eventually develop resistance to most of the treatment (Lebbink et al., 2017). Numerous scientists have created CRISPR technologies that can target host DNA-integrated HIV-1 genes to eradicate the infection in situ (Lebbink, 2017; Wang, 2016). Researchers employed a CRISPR system with one guide RNA or two guide RNAs at the same time to investigate the human cell line's HIV-1 genome. Rapid viral resistance resulted from using a single guide RNA, but using two guide RNAs for dual targeting completely suppressed viral replication and left no trace of viral escape (Lebbink et al., 2017). Furthermore, the CRISPR/Cas9 system's continuous co-expression in HIV-1-erdicated cells stopped reinfection (Kaminski, 2016; Bella, 2018). HIV-1 was recently eliminated from a humanized mouse model of the illness using CRISPR/Cas9 system (Dash et al., 2019). After sequencing over 100 predicted off-target locations, no off-target effects were found. Lastly, CRISPR/Cas9 technology was employed in an ex vivo therapeutic strategy to alter CD4+ germline cells' CCR5 receptor, rendering them impervious to HIV infection from the start (Li et al., 2015). This makes it possible for HIV-1 patients to receive ex vivo treatment, which entails taking the patient's own T cells, altering them externally using CRISPR, and then returning them to provide them with a healthy immune system that is resistant to HIV-1 (Savić, 2016; Hirakawa, 2020). Pigs' porcine endogenous retroviruses (PERVs) have also been eliminated using CRISPR/Cas9 systems. A significant obstacle to xenotransplantation, the transplantation of pig organs to human recipients, is PERVs, which are viruses that have been incorporated into the pig genome. CRISPR/Cas9 technologies targeted the highly preserved PERV polymerase gene to remove PERV from porcine cell lines and render pig organ PERV-free. The PERV viral burden was subsequently reduced by a factor of 1000 (Yang et al., 2015). Later, PERV-free pigs that might be utilized to develop organs for xenotransplantation were created using somatic cell nuclear transfer (Niu et al., 2017).

Utilizing CRISPR/Cas9 systems target several additional human DNA viruses. Epstein Barr Virus (EBV) (Yuen, 2023; Hsu JL, 2000; Kanda, 2016), Herpes simplex virus-1 (HSV-1) (Van Diemen, 2016; Lin, 2016), human papilloma virus (HPV) (Kennedy, 2014; Hu, 2014; Yoshinouchi, 2003), human cytomegalovirus (CMV) (Van Diemen et al., 2016), and JC polyomavirus (Chou et al., 2016) are among them. The RNA genomes of many significant human infections, however, include rotaviruses, flaviviruses (Zika virus, West Nile virus, yellow fever, and dengue) and coronaviruses (SARS-CoV, MERS-CoV, and SARS-CoV-2). Two RNA-sequence targeting CRISPR methods have recently been developed. One such method uses a special DNA oligomer with the PAM sequence and the Cas9 protein, which has a specific inherent affinity for RNA. The double-stranded target required for Cas9 cleavage is formed when the PAM oligomer attaches to the target RNA sequence at the intrinsic PAM sequence (Butiuc-Keul, 2022; O'Connell, 2014). Cas13 proteins are a class of RNA-targeted nucleases used in a second RNA-modifying CRISPR system (Faure, 2019; Shmakov, 2017; Konermann, 2018). This tactic has been suggested as a treatment for the virus that causes Coronavirus 2019 (COVID-19) SARS-CoV-2. Under the direction of a tissue-specific promoter, the idea involves introducing a CRISPR/Cas13 system into airway epithelial cells in addition to guide RNAs that target the SARS-CoV-2 virus's replicase-transcriptase and spike protein genes. Delivery would occur through the aerosolization of a modified adeno-associated virus, a non-pathogenic human virus that targets the airway epithelium. This approach was recently evaluated in airway epithelial cells, demonstrating efficacy in cleaving produced SARS-CoV-2 RNA fragments (Nguyen et al., 2020).

CRISPR vs Traditional Methods

Conventional antiviral therapies, including pharmaceuticals and vaccines, along with prior gene editing technologies such as TALENs and zinc finger nucleases (ZFNs), have restricted applicability; however, anti-retroviral and nucleoside analogs manage these infections. The complete eradication of latent viral DNA is futile (Jezek et al., 1987). Vaccines can be efficacious in prevention; nevertheless, numerous individuals exhibit resistance due to viral changes. In comparison to CRISPR, ZFNs and TALENs exhibit less accuracy, although their impact on alternative targets is more significant. CRISPR is significant for its ability to specifically target and break viral genomes, presenting possibilities for eliminating latent infections, unresolved changes persist, including off-target effects, delivery complications, and immunological responses to CRISPR components. Unlike conventional medicines, CRISPR demonstrates greater precision and adaptability, although it requires further optimization for clinical application (De Clercq et al., 2009). Consequently, CRISPR would exceed conventional methods regarding specificity and adaptability, precisely targeting viral genomes with significantly reducing off-target effects compared to antiviral medications, which fre-

quently impact host cell processes (Wright et al., 2016). Moreover, in contrast to antiviral medication resistance, CRISPR is a more rigid and reliable RNA-guided system, resulting in a lower likelihood of error from escape mutations, hence enhancing its efficacy against developing viruses (Mali et al., 2013). While standard medicines offer antiviral suppression, the CRISPR approach facilitates genetic editing for latent viruses, hence enabling a functional cure for illnesses such as HIV and herpes. Technological developments in CRISPR have mitigated off-target concerns, rendering CRISPR a significantly more accurate and efficacious alternative to conventional medicines (Plummer et al., 2018).

CRISPR-Based Vaccines

Conventional vaccination entails the introduction of bacterial or viral antigens within the host, leading to the elicitation of a specific antibody response and/or a specific T cell reaction (Lynn, 2022; Gilbert, 2012). There is significant variation in the amount and caliber of antibodies produced by everyone, and the reaction is host-specific (Lynn et al., 2022). Furthermore, certain viruses, such HIV-1, elicit neutralizing antibodies in only a limited subset of persons, complicating vaccine development. A different approach involves modifying patient B cells to generate antibodies that neutralize. B cells from humans have been genetically engineered using CRISPR methods to generate broadly neutralizing antibodies (bNAb) specific to HIV-1, capable of suppressing HIV-1 viremia. Recent research produced bNAbs by modifying human patients activated B cells' heavy chain locus using CRISPR-mediated cleavage and homology-directed repair. The B cells were subsequently transplanted into mice, where they generated adequate antibody levels to establish protection (Hartweger et al., 2019).

Human Trials of CRISPR Therapeutics

Human patients are currently undergoing phase 1 trials with CRISPR systems. Ex vivo, CRISPR therapy, which involves harvesting the patient's cells (or donor cells), modifying them in a lab, and then reintroducing them into the patient, is used in most of these trials. CRISPR were used to CCR5-ablate hematopoietic progenitor cells in HIV-1 positive patient with acute lymphocytic leukemia to prevent HIV infection had a successful bone marrow transplant in 2019, according to a Chinese group (Xu et al., 2019). Following engraftment, CCR5 ablation was verified in some of the patient's T cells, and WGS indicated no evidence of off-target effects. T cells from three patients with resistant cancer, two with advanced myeloma, and one with liposarcoma were extracted, genetically altered using CRISPR to produce a T cell receptor specific to the NY ESO-1 tumor antigen, and then reintroduced into the patients in limited phase 1 research (Stadtmauer et al., 2020). For at least 100 days, the three patients' modified T cells' expression was verified. Two patients were still alive at the end of the ninth-month follow-up period, and one patient had passed away. Notably, chromosomal translocations were found in every transformed T cell population; however, the altered T cells did not appear to benefit from these translocations in terms of proliferation. Early in 2020, research on these in vivo CRISPR gene therapies in humans began. A CRISPR-based treatment system will be administered to pediatric patients with Leber congenital amaurosis, a congenital retinal condition, to correct the single bp mutation responsible for the condition, administered to the retina through a localized injection of an engineered adenovirus vector (Rasoulinejad et al., 2021). From the 1st phase 1 clinical trial in 2020 to the first CRISPR-gene editing method in human cells in 2013, the development of CRISPR therapies has advanced at an exceptionally quick pace. Considering the growing interest in CRISPR-oriented treatments for a range of illnesses, including multifactorial ailments like cancer and monogenic disorders like hemophilia and sickle cell anemia advancements are expected to proceed rapidly. The resolution of safety concerns would enhance the viability of employing CRISPR therapies in human patients affected with infectious diseases.

CONCLUSION

The identification of CRISPR repeats in bacteria was first seen as insignificant beyond the realm of microbiology. One of the most important discoveries in genetic engineering is CRISPR technology and is set to significantly influence various domains of clinical care. This paper delineates possible uses of CRISPR technology to treat infectious disorders, encompassing the creation of quick, cost-effective diagnostics, the treatment of bacterial illnesses resistant to antibiotics and the management of both acute and persistent viral infections. Diagnostics based on CRISPR and therapies

are expected to be integrated into clinical practice imminently, and doctors must recognize both their remarkable promise and associated hazards.

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