# A Review on CRISPR Technology as an Antiviral Tool

By

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Bachelor of Pharmacy (Hons.)

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## Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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# Approval

The project titled “A Review on CRISPR Technology as an Antiviral Tool” submitted by Sanjida Ahmed Srishti (17346004) of Summer, 2017 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelors of Pharmacy (Hons.) on December, 2021.

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## Ethics Statement

This study does not involve any kind of animal or human trial.

## Abstract

CRISPR/Cas system is a novel and highly adaptable technique that has evolved as a potential therapeutic and molecular diagnostic tool. As a promising novel antiviral approach, CRISPR technology is utilized in the degradation of human pathogenic viruses by either targeting the specific viral DNA directly or interfering with the spontaneous generation of host cellular components required for viral replication. This review focuses on the advancement in treatment and diagnosis of several infectious viral diseases such as Human immunodeficiency virus, Hepatitis B virus, Herpes simplex virus, SARS-CoV-2 and Human Papillomavirus through the application of revolutionary CRISPR/Cas system. It also discusses about various types of vectors that are utilized in the CRISPR/Cas system delivery. Moreover, it points out the prospects and challenges associated with the CRISPR/Cas system.



**Keywords:** CRISPR-Cas; genome editing; antiviral tool; vectors; SHERLOCK

# Dedication

***Dedicated to my beloved parents***

## Acknowledgement

All praises and glory to Almighty Allah (SWT) who has given me enormous courage, knowledge, wisdom and patience to carry out and complete this thesis. Peace and blessing of Allah be upon last Prophet Muhammad (Peace Be upon Him).

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## List of Acronyms

AAV Adeno Associated Virus

ABACAS Antibody and CAS fusion

AIOD CRISPR All-In-One Dual CRISPR-Cas12a

AML Acute myeloid leukemia

ART Antiretroviral treatment

CMV Cytomegalovirus

CONAN Cas3-Operated Nucleic Acid detection

CREST Cas 13-based rugged, equitable, scalable testing

CRISPR Clustered regularly interspaced short palindromic repeats

DETECTR DNA Endonuclease-Targeted CRISPR Trans Reporter

DIY bio Do-it-yourself biology

DNA Deoxyribonucleic acid

DSB Double strand break

FDA Food and Drug Administration

HBV Hepatitis B Virus

HD Histidine-Aspartate

HDR Homology-directed repair

HEPN Higher eukaryotes and prokaryotes nucleotide-binding

HIV Human Immunodeficiency Virus

HLA-A2 Human leukocyte antigen A-A2

HLA-B13 Human leukocyte antigen-B13

HPV Human Papillomavirus

HSK Herpetic stromal keratitis

HSV PML Herpes Simplex Virus promyelocytic leukemia

KSHV Kaposi's sarcoma herpesvirus

LAMP Loop-mediated amplification

LOD Limit of detection

LV Lentivirus

MERS CoV Middle East Respiratory Syndrome Coronavirus

MLV Moloney Murine Leukemia Virus

NER Naked eye readout

NGG Nucleobase-Guanine-Guanine

NHEJ Non-homologous end joining

PAC-MAN Prophylactic antiviral CRISPR in human cells

PAM Protospacer adjacent motif

PAMAM Poly-amidoamine

PCR Polymerase Chain Reaction

PDMAEMA Poly [2-(dimethyl amino) ethyl methacrylate]

PEI Polyethyleneimine

PLL Poly L-lysine

POCT Point-of-care testing

RNA Ribonucleic acid

RNP Ribonucleoprotein

RPA Recombinase polymerase amplification

rRT-RAA Real-time reverse transcription recombinase-aided amplification

RT-PCR Reverse transcription polymerase chain reaction

SARS COV 2 Severe acute respiratory syndrome coronavirus 2

SHERLOCK Specific High Sensitivity Enzymatic Reporter Unlocking

SIN Self-inactivating

SLN Solid nanomaterials

STOP SHERLOCK Testing in One Pot

TTTN Thymine-Thymine-Thymine-Nucleobase

UTM Universal transport media

VLP Virus-like particles

YMDD tyrosine-methionine-aspartate-aspartate

ZFN Zinc-finger nucleases

# Chapter 1

# Introduction

## 1.1 Background

The advancement of highly adaptable genome-editing techniques in recent years has given researchers the capacity to swiftly and affordably incorporate sequence-specific alterations to the genomes of different range of cells (Gaj et al., 2016) . The clustered regularly interspaced short palindromic repeats-CRISPR associated proteins known CRISPR/Cas systems are one such genome editing technique that since the last decade has aroused a lot of curiosity (Rutkauskas et al., 2017). The bacterial adaptive defense system is the origin from where the CRISPR/Cas genome editing technology developed (B. Zhang, 2021). From its surprising revelation as an adaptive immune response in bacteria to being one of the most versatile gene-editing techniques for having a broad range of applications, the CRISPR/Cas system has transformed every aspect of life science. In addition, virology is now a part of this ever-growing list of applications based on CRISPR/Cas. As witnessed earlier in history and even now with the ongoing pandemic, the potential of viruses to mutate frequently results in the evolution of variants which exhibit higher infectivity and immune resistance, thereby complicating the formulation of drugs specific to the variants. Numerous viruses transmitting in the species of animals and insects have been discovered by genomic identification; some of these microbial species have been proved to cause significant diseases and others may cause infections in humans. Antivirals that have been approved so far are primarily designed and need identification of the suitable targets of the viral or host protein. Numerous small-molecule inhibitors have been licensed for human use due to their efficacy. However, finding new inhibitors and repurposing those that have previously been evaluated is a

time-consuming and difficult procedure (Freije & Sabeti, 2021). CRISPR-Cas gene editing system has been extensively utilized for precise DNA or RNA target and cleavage or several biomedical applications including investigating the host–pathogen interaction, modifying the host genome for pathogen resistance, detecting infections, and directly invading pathogens for therapeutic purpose. According to the extensive understanding of the correlation of virus and host along with the fundamental rules of nucleic acid cleavage or gene expression in the CRISPR-Cas technique, this genome editing tool can be utilized by targeting both the viral DNA and the components of hosts for eliminating viral reservoirs and prevent viral proliferation or infection (S. Chen et al., 2018).

## 1.2 Objectives of the Study

From the revelation of DNA double helix, techniques for creating and modifying DNA have brought breakthroughs in science and CRISPR technology is one of the latest genome editing systems that holds a great therapeutic and diagnostic potential against viral diseases. CRISPR technology has sparked a revolution in which scientists all over the globe are utilizing the technology to develop new biological applications.

The objectives of this study are-

- to provide an overview of the CRISPR system as a breakthrough in genetic engineering method
- to provide an insight on how the CRISPR technology can replace conventional medicines with its genome editing potential and work as an antiviral tool
- to illustrate how the CRISPR technique is being utilized for treating different pathogenic viral diseases by genome editing, gene expression modulation, diagnostics and site-specific therapy

## 1.3 Rationale of the Study

Despite various viral breakouts in the last few decades, including the current catastrophic pandemic, diagnostic and medication technologies remain woefully inadequate. CRISPR technology has the potential to meet the criteria required to fight against various infectious diseases. The majority of currently available antiviral treatments for chronic viral infections have failed to establish clinical cure due to their inability to destroy the genome of a virus that has infected a host due to a latency stage where these viruses limit their function inside a host cell to avoid immune monitoring. CRISPR technology shows significant potential as a cure for persistent infection caused by pathogenic viruses in this regard. In the current circumstances, it is apparent that CRISPR-based technologies have enormous potential to contribute in the diagnosis and treatment as an antiviral tool. In the near future, these gene sequence specific therapies might be the key to long-term and successful treatment modality against several viral infections. Therefore, this review was conducted to provide an insight on the potential of CRISPR technology as an antiviral tool owing to its versatile nature, greater efficiency, unique mechanisms and selectivity in combating viral pathogens.

# Chapter 2

# Methodology

This study reviews the revolutionary CRISPR-Cas system along with its ground-breaking current applications in diagnosing and treating viral infections. For this extensive review, all the data and information were collected from authentic primary and secondary research articles indexed in Scopus, Science Direct, PubMed and Springer. The articles used to gather information for this review paper were published in journals like Nature, Frontiers, Immunity, Science, Cell, Nature Biotechnology, Virology, etc. For organizing the information in a systematic manner, an outline was formed at the beginning following which information was collected by scrutinizing various research papers. After going through a large number of research articles, 87 articles with relevant information were chosen for the study.

# Chapter 3

# CRISPR Technology

## 3.1 CRISPR Genome Editing System

CRISPR-Cas systems' RNA-guided nucleases are now regarded as among the most reliable methods for editing and modification of genome sequence. The initial clue of the presence of CRISPR was unraveled in 1987, when a unique repeating DNA sequence (later revealed as CRISPR) was identified in the genome of *Escherichia coli* during a study of specific genes associated in the metabolism of phosphate. Later, identical sequence patterns were discovered in a variety of different bacteria along with halophilic archaea, suggesting that such revolutionary invariant clusters of repetitive sequences might have a significant role to play in these prokaryotes. During the discovery of a connection among CRISPRs along with accompanying Cas proteins, these were initially believed to play a role in repairing DNA in Hyperthermophiles, and this was a key step towards functional evaluation of CRISPR-Cas systems (Ishino et al., 2018). The genome editing with CRISPR-Cas9 approach being part of the adaptive immune response protects archaea and bacteria against other infections. On invasion of nucleic acid from pathogens, the invading genome's target region is recognized by the system's sgRNA (single guide RNA). Also, the Cas9 nuclease functions like a set of scissors for splitting the double strands of DNA (C. Liu et al., 2017). The discovery in 2013 that the bacterial CRISPR-Cas9 adaptive immune response could be used for editing genes in human cells marked a significant advancement in genome editing technology. In contrast to genome editing techniques employing zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), Cas9 protein is guided by a complementary RNA to obtain sequence specificity for breaking foreign nucleic acid (Glass et al., 2018). Nevertheless, all of these nucleases cause the breakage of double strands of DNA which

can be fixed by either error-prone NHEJ (non-homologous end joining) or HDR (homology-directed repair) pathway as depicted in Figure 2. Unlike NHEJ, which involves insertions or deletions and disruption of genes at the target location, HDR can be used to incorporate a specific single or double stranded DNA template at the target location for accurate gene editing (Adli, 2018).

To allow therapeutic genetic modifications in eukaryotes, the CRISPR/Cas system has been designed into two parts which includes a single guide RNA (sgRNA or gRNA) and a Cas endonuclease combinedly resulting in the active ribonucleoprotein (RNP) complex as shown in Figure 1. The sgRNA sequence is made up of two domains: the spacer sequence (crRNA), which comprises of 20 nucleotide (nt) sequences complementary to the target DNA to be edited, and a backbone sequence (tracrRNA) which improves the binding specificity of Cas9 to the target site. Cas9 is an enzyme that is guided by sgRNA to the target site where it creates a cut in the DNA strand, which leads to the introduction of a mutation in the form of insertion, deletion or frameshift. For minimizing off-target activity of the gRNA, this process necessitates the presence of a particular sequence of nucleotides on the strand of DNA that is being targeted and referred to as the protospacer adjacent motif (PAM). Following that, the endonuclease splits the strands of DNA placed on the upstream of the protospacer adjacent motif sequence. Hence, the CRISPR-Cas target specificity is determined by RNA–DNA base pairing and the PAM sequence. Specific PAM sequences are required by different Cas proteins, such as 5′-NGG for endonuclease like Cas9 or 5′-TTTN for Cas12a /Cpf1 endonuclease obtained from *Streptococcus pyogenes*. Various types of Cas proteins also have unique cleavage techniques. For example, SpCas9 causes a blunt DSB (double strand break) three nucleotides above the PAM. By modifying the 20nt gRNA sequence,

any PAM site unique to the Cas protein of interest can be used to induce a double strand break (Wilbie et al., 2019).

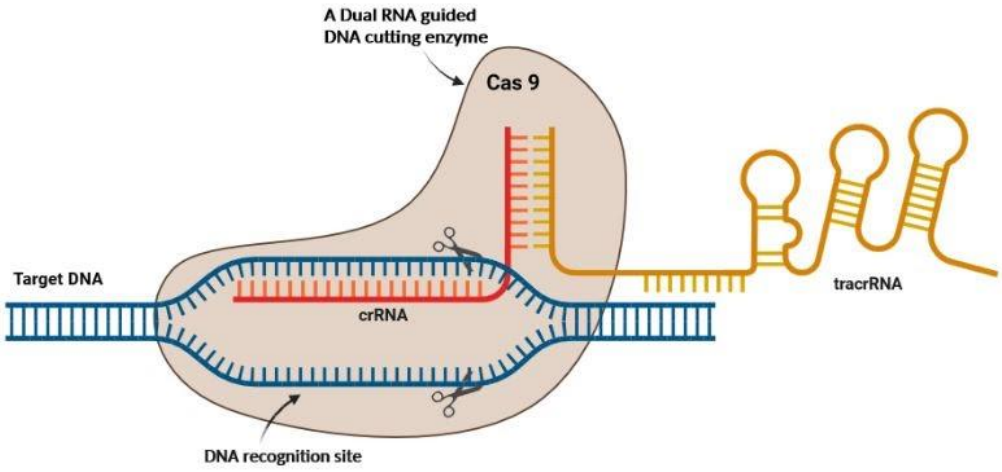


*Figure 1: CRISPR-Cas9 genome editing system. In this system, there are two short RNAs: tracrRNA and crRNA and both form a complex with a protein called Cas9. The tracrRNA helps to find the complementary DNA sequence of invading viruses. When the matching sequence of crRNA binds with the targeted DNA sequence inside the viral genome, Cas 9 breaks the target DNA rendering the virus inactive (Adapted from* Adli, 2018).

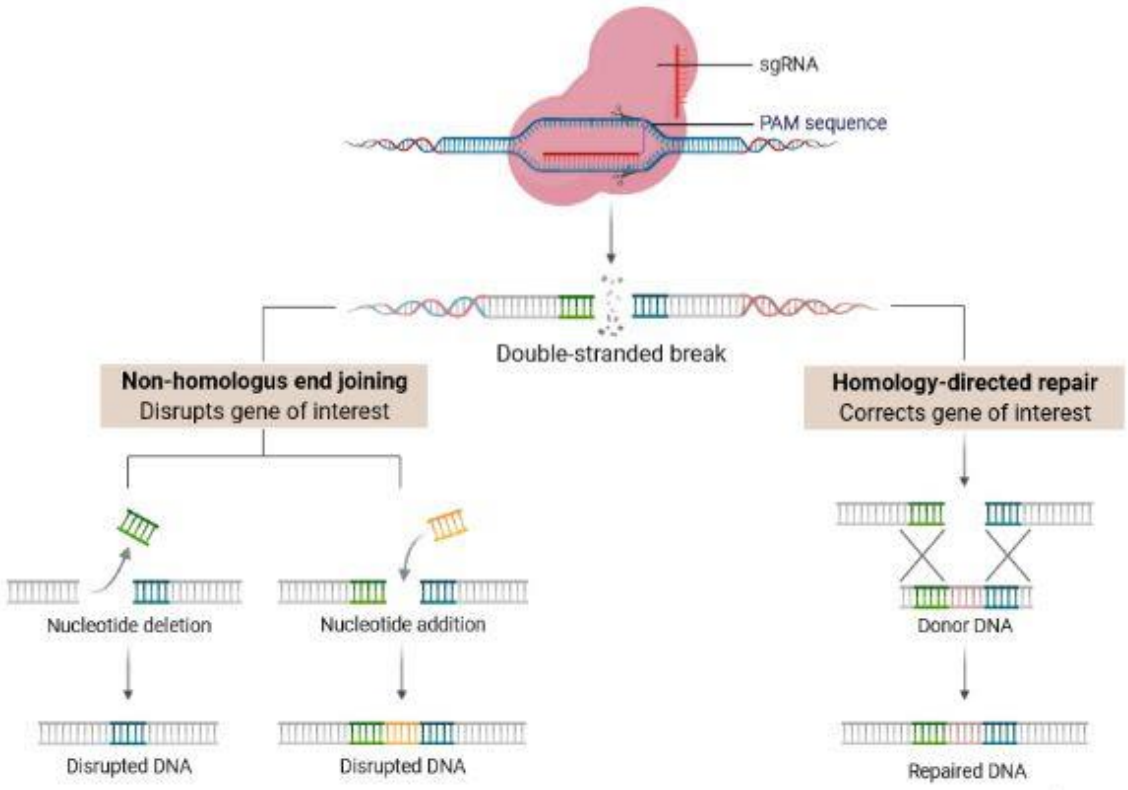


*Figure 2: DNA double stranded break repair through NHEJ and HDR. Non-homologous end joining repair pathway leads to removal or addition of nucleotides, potentially resulting in a gene knockdown or knockout or knock in. Homology guided repair results host DNA repair template insertion with a unique sequence surrounded by homology arms leads to a gene knock-in (Adapted from* Adli, 2018).

## 3.2 Different CRISPR-Cas Systems

Different bacteria and archaea may exhibit CRISPR-Cas systems of many varieties and these systems can be classified into Class 1 and Class 2. Class 1 is further divided into Type I, III and IV. Similarly, Type II, V, and VI falls under Class 2 (Figure 3). Notably, Types I, II, and V identify and split DNA, whereas type VI can modify RNA and type III can edit both the DNA and RNA.

However, whether the type IV system targets DNA or RNA is yet unclear (Z. Liu et al., 2020). CRISPR-Cas types are distinguished by their unique genes and the specific organization of their loci, protospacer adjacent motif (PAM) regions, Cas proteins of various sizes and splitting regions.

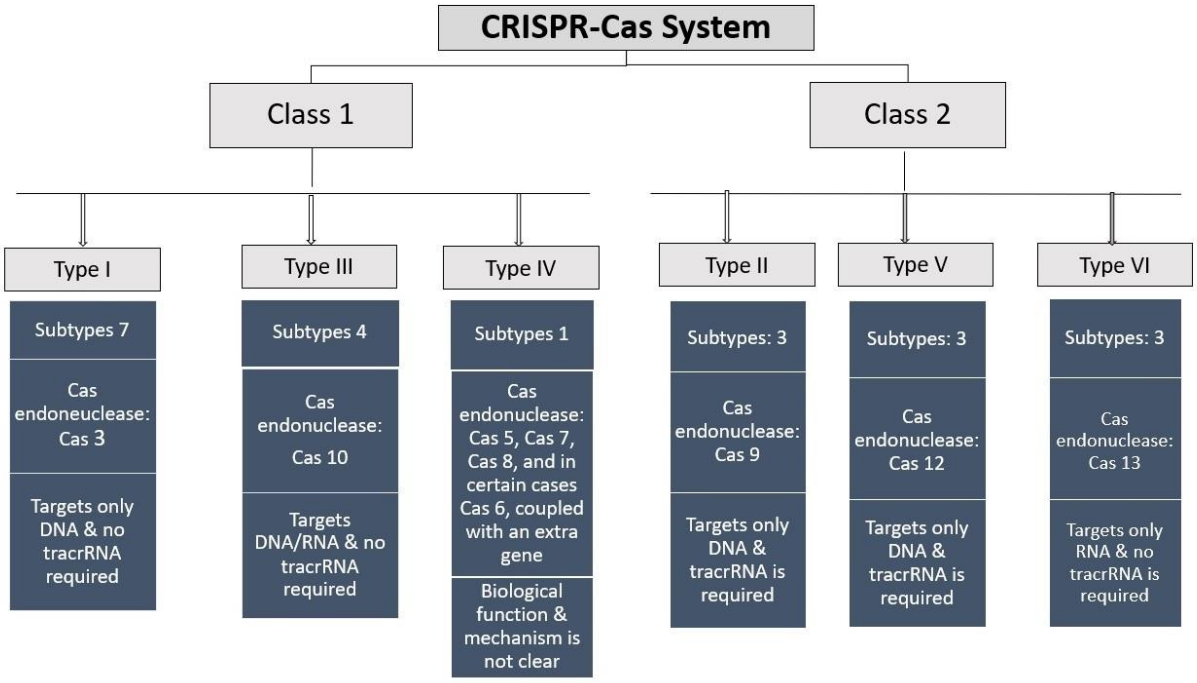


*Figure 3: Classification of CRISPR-Cas System (Adapted from Makarova & Koonin, n.d.-a)*

### 3.2.1 Type I CRISPR-Cas Systems

Type I CRISPR-Cas system includes type I loci that contain gene encoding for Cas 3 protein. This endonuclease consists of two parts- superfamily 2 helicases at the C-terminal of the protein and an HD domain at the N-terminal of the protein. The helicase is responsible for unfolding RNA–DNA compounds and double-stranded DNA (dsDNA) whereas the HD domain is engaged in the breakage of the target DNA strand (Makarova et al., 2015).

The CRISPR-Cas technique in the bacteria provides defense against the bacteriophage in three steps which include- spacer acquisition, crRNA processing, and interference. In spacer acquisition stage, when the bacteriophage first infects the bacterial cell and releases its viral genome, the bacterial cell chops up particular segments of viral genome and inserts those fragments into the CRISPR locus' spacer DNA which works as a memory for the bacteria on a second encounter with the same virus. Near the CRISPR locus are Cas genes which code for different Cas enzymes, resulting in a Cascade complex. This Cascade complex of different Cas proteins aids in cutting the viral genome upstream of PAM and inserting it into the spacer DNA during the spacer acquisition step on invasion of the virus for the first time. In the crRNA processing step, one of the two DNA strands containing the phage's genome is transcribed into mRNA followed by the formation of loops of the short palindromic repeats of CRISPR locus. The loops with the viral gene are cut by another group of Cas proteins- Cas6, Cas6e, and Cas6f resulting in crRNA formation. When the virus attacks for the second time, the Cascade-crRNA complex recognizes the viral genome sequence. The viral genome then binds to the complementary crRNA which activates and recruits Cas3 nuclease to cleave and degrade the viral genome. As a result, the virus can no longer replicate.

### 3.2.2 Type II CRISPR-Cas System

Type II CRISPR-Cas system includes type II loci that contain gene encoding for Cas9 protein that leads to double strand break of the viral DNA resulting in blunt ends i.e. break two strands of DNA at the same place. Cas9 enzyme has two domains- HNH and RuvC to create the DSB. Type II CRISPR-Cas system also provides defense against the bacteriophage in three steps which include- spacer acquisition, crRNA processing, and interference (Clark et al., 2019).

### 3.2.3 Type III CRISPR-Cas System

CRISPR-Cas technique of Type III includes type III loci that contain gene encoding for Cas10 protein that can cleave both DNA and RNA. Besides, Type III CRISPR-Cas system also provides defense against the bacteriophage in three steps which include- spacer acquisition, crRNA processing, and interference. It is the most commonly used CRISPR-Cas gene editing system.

Type III CRISPR-Cas systems differ considerably from type I systems in terms of content and structure. The heterogeneity is obtained by gene repetitions and removals, domain fusion and introduction as well as the presence of additional, poorly described regions that are probably engaged in effector complex molecules or related immunology. Two variations of type III (one of type III-A and one of type III-B) are very frequent. The cyclase region of Cas10 is clearly inactivated in type III-B variations, but the variants of type III-A generally include a gene known as Cas10 without the HD domain as well as an unidentified gene similar to all. In most cases, both type III variations as well as various systems of CRISPR-Cas can be situated in a genome(Makarova & Koonin, n.d.-b).

### 3.2.4 Type IV CRISPR-Cas System

CRISPR-Cas systems of type IV are a simple form distinct from those transmitted by Tn7-like transposons. Plasmids and, in rare cases, prophages from different bacteria have type IV loci. Limited exceptions apart, type IV systems lack an adaptation unit and also composed of cas5, cas7, cas8, and in certain cases, cas6, coupled with an extra gene that generates either a DNA helicase from DinG family or an unidentified small protein in various type IV variations. As CRISPR arrays are rare at type IV loci, they are more likely to appear in trans arrays, which are placed in another

area of the similar plasmid distant from or on the host genome. It's unclear what type IV CRISPR-Cas systems do. However, given that they are almost exclusively found on plasmids, it's reasonable to assume they aid stability and improve movement of plasmids through undiscovered ways (Макаров & Makarov, 2019).

### 3.2.5 Type V CRISPR-Cas System

The CRISPR-Cas technique of type V is composed of three components: the effector protein module, the acquisition module as well as the CRISPR array. The type V effector proteins have a uniform RuvC-like endonuclease (RuvC) region at the C-terminus. Moreover, the type V technique is subsequently classified into several subtypes based on the different CRISPR effectors, such as types V-A through V-I, type V-K, type V-U, and CRISPR–CasΦ (Hajizadeh Dastjerdi et al., 2019; Pausch et al., 2020). The related effector proteins in these subtype systems have demonstrated a range of activities, with some acting not just on double-stranded DNA (dsDNA), as well as on single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA). Recent research has focused on the CRISPR–Cas method of type V due to its multi-functionality. The hallmark effector protein Cas12a (previously named Cpf1) is found in type V-A, which was discovered in the human pathogenic bacteria *Francisella tularensis* in 2013. Cas12a, which is controlled by gRNA, utilizes a single RuvC nuclease region to split the non-complementary and complementary strands of the specifically targeted DNA sequence. This is in contrast to the type II CRISPR enzyme Cas9, which utilizes the nuclease domains like HNH and the RuvC to cleave the two strands of the targeted DNA region. The C-terminus of the type V CRISPR system distinguishes it from others, which has just one RuvC endonuclease domain (Tong et al., 2021).

### 3.2.6 Type VI CRISPR-Cas System

CRISPR-Cas method of type VI, which only target RNA that are single-stranded (ssRNA), have just been identified. To date, four subtypes (A–D) have been discovered, with subtypes VI(A), VI(B), and VI(D) being efficiently described along with their corresponding effectors, Cas-13a, Cas-13b, and Cas-13d, respectively. Effectors like Cas 13 are all crRNA-guided RNases along with two separate catalytic sites (Daniel E Shumer, 2017; Shmakov et al., 2016).

The first catalytic centre handles pre-crRNA, while the second is produced by two R-X4-H motifs found in nucleotide-binding (HEPN) domains present in higher eukaryotes and prokaryotes that facilitate ssRNA cleavage. Unlike Cas9, which splits crRNA spacers complementing dsDNA sequences (e.g. target DNA) exclusively in cis, the active Cas13-crRNA interference group splits non-specifically the corresponding ssRNA sequence linked to crRNA in cis, as well as any other RNA encountered (including host and viral RNA) in trans (also known as collateral or bystander cleavage). Cleavage occurs primarily in structurally accessible areas of secondary structures of RNA, often at uridine (U) or adenosine (A). The effectors of Cas13 have already been utilised as methods for the modification of RNA due to their strong activity in mammalian cells, despite their practical applicability (Daniel E Shumer, 2017; Shmakov et al., 2016; Yan et al., 2019).

Due to their effective, highly precise, and programmable RNA-targeting characteristics, as well as autonomous processing of pre-crRNA, type VI CRISPR-Cas systems drew a lot of interest in the years after their invention, biochemical and structural identification. Transcriptome alteration by Cas13 effectors offers many benefits over genome editing based on CRISPR: It is secure since it is transitory and reversible, the degree of editing is depending on dose as well as may be adjusted to meet different goals. Furthermore, CRISPR-Cas systems of type VI show collateral ssRNA

cleavage efficiency in bacteria and *in vitro*, which can be utilized in diagnosis. As a result, a growing number of experiments have been conducted with the goal of developing type VI CRISPR-Cas systems into essential components for fundamental studies, biotechnology and medicines (Perčulija et al., 2021).

# Chapter 4

# Strategies for CRISPR-Cas System Delivery

## 4.1 Viral Vectors

Since mammalian gene editing is still in its early stages of development, it's remarkable how many studies have already demonstrated the power and potential of CRISPR in conjunction with viral vector delivery methods. To date, four important types of viral vectors including adeno-associated viruses (AAVs), adenoviruses, retroviruses, and lentiviruses, have been employed as vectors for Cas9 and/or gRNA delivery (Schmidt & Siebert, 2001).

### 4.1.1 Adeno-associated Virus

Adeno-associated virus vectors are non-pathogenic, powerful, and easy to construct. Adeno-associated virus (AAV) is capable of delivering the two key CRISPR elements Cas9 and RNA either independently or in combination (Grimm et al., 2008). Cas proteins that are relatively small AAV is a non-enveloped virus having a 25-nanometer diameter. Several decades of research showed that the AAV vector can occupy a genome of size restricted to only 5 kb, leaving the transgenic cassette with a maximum size of 4.7 kb. Cas proteins have been found to be more compatible with AAV delivery, allowing more vector design possibilities such as wider promoter choices and a more efficient delivery method (D. Wang et al., 2020). For instance, the gene encoding for Cas9 from *Staphylococcus aureus* (SauCas9) has a length of 3.2 kb and allows incorporation of a maximum of two sgRNAs with the Cas9 gene in a particular AAV vector (Ran et al., 2015). An ‘‘all-in-one" AAV8 vector that not only accommodates SauCas9 gene and two sgRNAs but also carries a self-linearizing repairing template was recently discovered. This vector has been demonstrated to repair a Fah (fumarylacetoacetate hydrolase) mutation in mice, allowing

it to cure inherited tyrosinemia type 1 (Krooss et al., 2020). Despite the fact that there are several varieties in viral vectors whereas adeno-associated viruses have been mostly employed for CRISPR genome engineering in cells and animal studies. This implies that AAV is an excellent toolbox for CRISPR applications in a broad number of cell types, both *in vitro* and *in vivo* (Senís et al., 2014). Due to constrained packing capacity, multiple vectors are often required. AAVs are widely used for a variety of applications. For example, AAVs have previously been authorized for use in a variety of human clinical studies involving gene augmentation treatments because of their great profile for safety and therapeutic possibilities. Additionally, in higher doses, moderate toxicity is generated in animal models. After transduction, AAV DNA stays mainly episomal, with the exception of combining into hotspots in the DNA of mitochondria and a particular place on human chromosome 19 can be referred as adeno-associated virus integration site 1 (AAVS1). At the moment, both integration locations are deemed safe and do not promote tumorigenesis. Additionally, there are a significant variety of serotypes of AAV that enable the delivery of tissue-specific genes. Since some serotypes have been proven to be appropriate for CRISPR-editing in particular tissues (Kaeppel et al., 2013). Recombinant adeno-associated virus causes a very low but significant innate immune response when compared to other viral vectors. The innate immune system is the body's initial protective barrier towards viral infection. Pattern recognition receptors (PRRs) sense pathogen-associated molecular patterns (PAMPs) on invading virions or viral nucleic acids and detect them. A signal transduction event is triggered to restrict viral propagation and to trigger adaptive immune responses specific to pathogens by these sensors (Nidetz et al., 2020). The immune system, on the other hand, continues to pose substantial hurdles in terms of safety and effectiveness. Even while temporary immunosuppressive may suppress CD8+ T cell responses to the capsid, pre-existing neutralizing antibodies (NABs) pose a challenge during the

first vector administration, and immunological responses to a given vector can lead to fast seroconversion, which makes vector re-administration challenging. However, as our understanding of AAV biology grows, we may rationally design or chemically modify capsids to prevent them from being detected. On the other hand, directed evolution may swiftly produce variants that are immune to neutralizing antibodies, and such choices can even be coupled with evolution for targeted distribution to specific tissues and cells *in vivo*. Adenovirus vectors may thus depend on a broad and developing toolbox, which is rapidly being applied to various patients and products (Barnes et al., 2019).

### 4.1.2 Adenovirus

Current genetic transmission vehicles for mammalian cells include adenovirus (Ad) vectors, which are incredibly convenient. As adenoviruses are non-encapsulated dsDNA viruses with a genome about less than 36 kb in size, viral particles of it do not incorporate into the host genome, eliminating the possibility of oncogenicity or genotoxicity. Their capacity to simultaneously transduce both dividing and non-dividing cells enables gene transfer *in vivo*, but the vast range of cell categories infected through adenovirus needs targeting, especially if the gene which is transfected is harmful when expressed in non-targeted tissues (Boucher et al., 2020; Sadeghi & Hitt, 2005). Recombinant adenovirus vectors are still the most popular choice for genome editing and functional genomic research as they are easy to build, can be made in large quantities, and have excellent transduction efficiency. These characteristics, along with proven safety profile of adenovirus in clinical studies, make it an appropriate choice for *in vivo* delivery of revolutionary genome editing machinery like CRISPR-Cas. An effective method for controlling how adenovirus particles behave *in vivo* after injection is through the modification of the protein capsid. This has

led in the invention of vectors that have resolved some of the problems associated with *in vivo* adenovirus delivery, including 1) viral sequestration in the liver, 2) restricted transduction of cells lacking the viral receptor; and 3) pre-existing neutralizing antibodies to adenovirus (Liebert, 2004).

### 4.1.3 Retrovirus

Retroviruses are RNA viruses with a positive sense that need reverse transcriptase to transform their RNA into DNA to be integrated into the host genome. A retrovirus contains four genes in its canonical genome- pol gene, env gene, pro gene and gag gene. The pol gene includes gene coding for reverse transcriptase enzyme, RNaseH also contains an integrase gene. The gag gene generates the structural polyprotein, whereas env gene produces envelope proteins that aid in viral attachment and tropism determination. The pro gene expresses protease required for maturity of the viral particle by converting immature polyproteins into functional elements, the earliest retroviral vectors used to transduce mammalian cells were actually based on the Moloney murine leukemia virus (MLV), and they could only successfully transduce dividing cells. Furthermore, these vectors were improved to only integrate transgenes of choice rather than viral genes, which was an important step because accurate gene integration is required for targeted gene therapy and genome alteration. As a result, with further research into retroviral gene delivery, viral vectors originating from the human immunodeficiency virus (HIV) were developed, which had the benefit of spreading viral tropism into non-dividing cells. For this reason, HIV-based vectors are commonly called "lentiviral vectors" since they have the potential to transduce non-dividing cells. Retrovirus-based vectors have had an influence on the treatment of various human diseases, and their usage may expand as vectors become safer. A fundamental research demonstrated the efficacy of a self-inactivating (SIN) -retrovirus vector in the treatment of X-SCID (DiCarlo et al.,

2017). As a result, current retro- and lentiviral vectors generally feature "self-inactivating" or "SIN" genomes in which the viral long terminal repeat eliminates its gene transcription in the transduced cell, which reduces concerns about the replication of viable viruses and increases safety. Regardless of their genetic content, all recombinant viruses have one thing in common: transmission into immunocompetent animals or human beings will inevitably elicit a humoral immune response mostly against proteins in the viral shell (F. Schmidt & Grimm, 2015).

### 4.1.4 Lentivirus

The lentivirus (LV) is a spherical virus with a single-stranded (ss) RNA which is proficient for both proliferating and non-dividing cells in the same way as adenovirus does. The most recent lentivirus vector systems divide important genes into three plasmids, minimizing the chance of active virus particles being produced within cells. Moreover, the lentivirus vector has a packaging ability of about 8 kb. One significant benefit of the lentivirus is its ability to be pseudo typed along with other viral proteins. This permits the cellular tropism of the LV to be engineered and modified (C. L. Xu et al., n.d.) Furthermore, LV vectors contain no viral genes and do not elicit an immunological response. Because most labs lack the ability to manufacture non-integrating lentiviruses. LV vectors are utilized less frequently than adeno associated virus and adenovirus vectors. Besides, LVs are most usually employed to develop disease models. Aside from genetic testing, animal models were created using LV-delivered CRISPR-Cas9. Aside from genetic testing, animal models were created using LV-delivered CRISPR-Cas9. For instance, a mouse model with acute myeloid leukemia (AML) developed in a single hematopoietic stem cell by altering up to five genes to simulate the genetic complexities of malignancy (Heckl et al., 2014).

## 4.2 Non-viral Physical Vectors

CRISPR-Cas9 can be delivered directly to cells by physical transfection. The physical transfection uses forces mechanically or electrically to produce transitory pores within the cell membrane, allowing target molecules to enter the cell more easily. Moreover, the use of vectors isn't required for physical transfection. As a result, unlike viral vectors, cargo size is virtually unlimited, and unlike chemical vectors, the rate-limiting process is independent of cell endocytosis.

### 4.2.1 Microinjection

A conventional model for mechanical transfection, called microinjection, involves injecting genes directly into cells via micro capillaries. Microinjection allows injecting genetic materials directly into the nucleus, allowing for fast expression of genes. When it comes to single-cell applications, such as germline editing, microinjection is indeed the way to go. CRISPR delivery through microinjection has sped up the development of model organisms exhibiting desired characteristics. By introducing gRNA directing the myostatin-encoding gene along with Cas9 mRNA into the cytoplasmic matrix of sheep zygote, scientists were able to develop sheep with muscular growth inhibitory genes knocked out. Researchers have successfully injected CRISPR into a wide range of organisms, including insect vectors, zebrafish, rodents and pigs, with great success. This technique is highly effective but has poor throughput, and an expert technician is necessary to properly inject the cell so that they stay viable (Fajrial et al., 2020).

### 4.2.2 Electroporation

Generation of a relatively competent cell (one with improved membrane permeability) by electric current is defined as electroporation. The short-term elevation in cell membrane permeability

permits RNA, DNA, and specific proteins to enter target cells, allowing them to function effectively. In recent years, electroporation has been identified as a viable method for delivering CRISPR-Cas9 systems to cells. There has been research that has used electroporation as a delivery method for genome editing. CRISPR-Cas9 plasmid systems, the Cas9 protein-and-sgRNA complex, Cas9 mRNA and single guide RNA are all electroporated by electroporation (Karimian et al., 2019).

## 4.3 Non-viral Chemical Vectors

### 4.3.1 Lipid Based Vector

To date, gene transfer through lipids is one of the oldest and most effective techniques for gene therapy. As cationic lipid-based vectors, liposomes and solid nanomaterials (SLN) are routinely utilized. These liposomes have become one of the most popular gene carriers due to their increased biochemical attributes and mild immunogenicity. SLNs are made up of high-melting-point lipids that have a solid core and are coated with surfactants to prevent them from melting. They are non-viral gene transfer carriers that can be administered systemically. Solid lipid nanoparticles offer greater storage stability and can be sterilized and lyophilized, allowing them to be used as non-viral carriers in a wide range of applications. As the most conventional non-viral gene carriers, cationic liposomes were the first non-viral delivering vectors used in clinical studies. Lipid nanoparticles aid in the transfer of CRISPR/Cas9 components into cells and can help shield the payload from degradation to some extent. Commercially Lipofectamine$^{TM}$ CRISPRMAX$^{TM}$ Cas9 (Thermo Fisher Scientific) is a widely manufactured lipid nanoparticle transfection reagent for delivering CRISPR-Cas9 ribonucleoproteins. There are several reports of liposome-based gene therapy that have progressed to clinical trials. As part of a phase I research, DC-Chol/DOPE

cationic liposomes were used to transport the human HLA-A2, HLA-B13, and murine H-2K genes to patients with various cancers, including leukemia. Specifically, *in situ* gene therapy elicited a robust immunological response, but no major adverse effects were observed. With the treatment of the HLA-A2-DNA liposomes, two out of eight patients had a full remission of cutaneous lesions (Li et al., 2015).

### 4.3.2 Polymer Based Vectors

For transferring the elements of CRISPR/Cas9, polymers have been widely researched as non-viral vectors, primarily because of their biocompatibility, adaptability, and simplicity, among other factors. The intrinsic versatility of the polymeric matrix makes it an intriguing type of delivery medium for CRISPR/Cas9 components. Through the rational development of molecular structure polymeric carriers, as contrasted by lipids carriers, can avoid instability issues of serum (X. Xu et al., 2019). Cationic polymers including polyethyleneimine (PEI), poly [2-(dimethyl amino) ethyl methacrylate] (PDMAEMA), poly(L-lysine) (PLL), and poly-amidoamine (PAMAM) dendrimers have received substantial interest in gene therapy due to their reduced immunogenicity and ease of wide manufacturing and functionalization (Li et al., 2015).

### 4.3.3 Chitosan System Based Vector

Chitosan-based vectors have arisen as possible non-viral vectors capable of securely delivering genetic components such as pDNA (parental plasmid DNA), siRNA (small interfering RNA) and ODNs (oligodeoxynucleotide). Chitosan is basically a polysaccharide made up of glucosamine as well as N-acetylglucosamine that is made from chitin that has been partially deacetylated. Through the use of different cell-targeting ligand conjugates, chitosan complexes may be delivered to

certain types of cells. Chitosan has several features, including reduced toxicity, mild immunogenicity, excellent biocompatibility, and a significant cationic charge. Chitosan's positive charge enables it to easily form polyelectrolyte compounds with negatively charged nucleotides via electrostatic interactions. Nevertheless, its clinical use is still limited for poor specificity and transfection effectiveness (Nimesh, 2013). A formulation containing the nonionic poloxamer CRL1005 and the cationic surfactant benzalkonium chloride has recently entered clinical trials. In a phase II/III trial, this formulation is being investigated as a genetic vaccination to minimize CMV infection in patients having allogeneic hematopoietic cell transplant (Li et al., 2015).

## 4.4 Extracellular Vesicles as Vector

In order to generate extracellular vesicles, the viral envelope and/or viral basic proteins must be generated and self-assembled. Besides, viral envelopes and structural proteins are referred to as virus-like particles (VLPs). The protein of choice is coupled to the Gag polyprotein, allowing both to be integrated into a particle at the same time. An enzyme from Pol facilitates Gag polyprotein degradation during viral maturation, which then releases a protein of choice that may be delivered to cells in the body. Although extracellular vesicles (EVs) do not contain any viral genome, as opposed to lentiviruses (LVs), which do contain viral genome. As a result, extracellular vesicles are not incorporated into the genomes of their hosts and do not reproduce on their own. Furthermore, EVs' better safety characteristic enables them a safe form of viral delivery. It is possible that CRISPR-Cas9 and the production of gRNA via viral carriers may generate off-target mutations and immunogenicity. As a result, a transitory delivery method is required for therapeutic genome editing purposes. Cas9 protein is recruited into extracellular nanovesicles by chemical mediated dimerization, and subsequently a viral RNA packaging signal and two self-cleaving

riboswitches attach and deliver sgRNA into nanovesicles. As a result, precise CRISPR/Cas exposition by EVs in cells significantly lowers the possibility of off-target effects caused by long-term Cas9 expression. In addition, the generation of EVs is easy and cost-effective, as it simply requires the usual transfection of plasmids into packaging cells. In future, using vesicles, multiplexed gene editing is possible. During the production of vesicles, they don't require protease cleavage. Therefore, it is not constrained by protease-mediated protein denaturation or the competitiveness between Gag and Gag-Cas9 proteins during packing, which are the drawbacks of the VLP-based method (Gee et al., 2020; Yip, 2020).

# Chapter 5

# CRISPR-Cas System for Combating Human Pathogenic Viruses

## 5.1 RNA Guided CRISPR-Cas9 System

The RNA-guided attachment and breakage of nucleic acids through CRISPR–Cas method is a unique characteristic of bacterial and archaeal adaptive defense systems against all viruses and plasmids. Cas9 and other single-subunit effector enzymes from CRISPR–Cas methods have been extensively embraced for scientific study and therapeutic human genome editing applications for their unique programmable capacity to cut particular sequences of DNA and RNA (J. S. Chen & Doudna, 2017). In the mammalian cells, reconstitution of the functionality of RNA-guided nuclease by CRISPR-Cas system is performed through heterologous production of human codon–optimized Cas9 and the required RNA elements. Cas9 nuclease, crRNA array encoding guide RNAs (tracrRNA) and auxiliary trans-activating CRISPR (tracrRNA) allow processing of Type II CRISPR array into distinct components, making it one of the most well-characterized CRISPR systems (Ran et al., 2013). CRISPR method originated as an adaptive immune response of bacteria where a phage DNA is cleaved by nuclease (e.g., Cas9) to provide resistance to infection. Because of its ability to couple bases, Cas9 may target a specific genomic region in order to cleave. Moreover, a particular small sequence of DNA that is targeted, known as the protospacer adjacent motif (PAM) which is required for the selectivity of Cas9 activity, the CRISPR-Cas9 has been used as a flexible method for guided genome engineering along with the aforementioned restrictions, enables the specific targeting of any specific chromosomal site of interest. SpyCas9 (*Streptococcus pyogenes* Cas9) is a big nuclease along with 1368 residues of amino acid that is commonly utilized in genome-editing applications. SpyCas9's potential advantage s comprise a

relatively small 5′-NGG-3′ PAM as well as a remarkable editing ability. Though the selective feature of SpyCas9 in various applications has been questioned. SpyCas9's above-mentioned characteristics have caused it to become considered as the most preferred CRISPR nucleases in the application area of clinical gene therapy (M. J. Schmidt et al., 2021). Developing CRISPR-Cas9 system guided by RNA to directly target HIV-1 regulatory genes might be a viable solution. A lentiviral vector form of transduction is a technique in which the gRNA inserted into lentiCRISPRv2 is utilized to selectively target the regulatory sequences tat and rev. The tool was effectively transduced into 293T and HeLa cell lines, eliminating the persistently expressing Tat and Rev proteins. As a consequence, the tat and rev genes' function assays indicated a substantial decrease in HIV-1 promoter-driven luciferase production and suppression of gp120 (glycoprotein expressed on the surface of the HIV envelope) function. Gene editing with CRISPR-Cas9 was also performed on herpes simplex virus 1 and 2 (HSV-1 and HSV-2), Epstein-Barr virus, cytomegalovirus and Kaposi"s sarcoma herpesvirus, as well as other viruses like Kaposi’s sarcoma herpesvirus (KSHV) (Bhattacharjee et al., 2019).

## 5.2 Collateral Cleavage Method by Cas12 and Cas13

Class 2 systems are indeed convenient to edit the mammalian systems since nucleic acid cleavage is accomplished by a single, multidomain effector protein. Depending on the Cas effector protein that facilitates interference. Class 2 system is subdivided into type II, V, and VI (Makarova et al., 2020). The type V and type VI Cas proteins known as Cas12 and Cas13 were further detected, revealing a distinct, key target, non-specific activity in cleaving known as collateral cleavage (Abudayyeh et al., 2016). Single RuvC endonuclease domain in the Cas12 gene cleaves either dsDNA or ssDNA. The cleavage through Cas12 generates staggered dsDNA fragments, far from

the PAM, also with a 4 to 5 nucleotide overhang (Zetsche et al., 2015). While most class 2 systems target DNA, the newly identified type VI effector Cas13 selectively cleaves single-stranded RNA (ssRNA). Cas13 was first thought to be an RNA-targeting protein due to the presence of two distinct higher prokaryotic and eukaryotic nucleotide (HEPN)-binding domains. Domains involved with antiviral and abortive infection processes are found in class 1 Cas effectors that target RNA(Freije & Sabeti, 2021) . Cas13, in contrast to Cas9 and Cas12, targets RNA and the sites of cleavage are not crRNA-specific. Although Cas13 RNA cleavage does not necessitate a PAM, preliminary research found contradictory evidence for a necessary sequence motif next to the target region, known as the protospacer flanking site (PFS). Cleavage activity was firstly observed in Cas13. Cleavage of trans-cleaved ssRNA did not need to be complementary to the supplied Cas13 crRNA after target identification by Cas13 (Abudayyeh et al., 2016). Since its first discovery, the activity of collateral cleavage was detected for various Cas12 subtypes. While a DNA target was present, the Cas12 was demonstrated to split ssDNA in trans. This cleaved ssDNA did not necessitate a Cas12 crRNA-complementary sequence. Neither the Cas13 nor Cas12 showed any collateral cleavage activity without complementary nucleic acids target. Following the revelation of collateral cleavage activity, several CRISPR-based diagnostics and virus detection methods were developed. A wide spectrum of viral targets has been demonstrated to be detected using Cas13- and Cas12-based diagnostics with single-plex tests (Freije & Sabeti, 2021).

## 5.3 CRISPR Nucleic Acid Based Approaches

Among the various antiviral defense mechanisms found in prokaryotes, CRISPR-Cas systems are the solely known RNA-programmed routes for recognizing and destroying various viral pathogens. The most common and varied of these adaptive immune systems, Class 1 CRISPR-Cas

systems, employ an RNA programmed complex of multi-protein to identify and destroy invading nucleic acids (T. Y. Liu & Doudna, 2020).

Type I of CRISPR-Cas systems in phages or plasmids, degrade homologous sequences of double-stranded DNA. The entire targeting process consists of two key steps: 1) Through the surveillance group, identification of a similar target in the foreign DNA and 2) Scission of the complementary target through Cas3. A specific protein that is actively recruited in trans contains a Superfamily 2 (SF2) helicase as well as a histidine-aspartate (HD) nuclease domain. For target identification, the involvement of a (PAM) Protospacer adjacent motif (which permits host organism to avoid autoimmunity) is required along with the homology between the crRNA and the target (Gleditzsch et al., 2019; Hochstrasser et al., 2014).

CRISPR-Cas technique of type III is known as the most evolutionary primitive method and commonly found in prokaryotic. For antiviral nuclease activation in trans, enzymatic domains of their ligand like effector molecules cut RNA and ssDNA along with generating molecules like second messenger. As a result, their effector complexes organize a robust, multi-pronged defense targeting foreign genetic components such as DNA and RNA viruses, plasmids, and giant bacteriophages. Furthermore, Type III CRISPR method is classified in six subtypes (III-A through III-F), where Type III-A and III-B systems are known as most prevalent. The valid investigation of systems has provided overview of this evolutionary history of CRISPR-Cas immunity as well as unexpected similarities with the immune response of other prokaryotes and eukaryotes (T. Y. Liu & Doudna, 2020).

# Chapter 6

## CRISPR-Cas System Based Diagnosis of Human Viral Infections

DNA detection technique that uses RNA-guided CRISPR/Cas nuclease has subsequently shown tremendous possibilities for the establishment of next-generation diagnosis technique because of its great delicacy and dependability. Some Cas nucleases, for example, Cas12a, Cas12b, and Cas13a, have significant collateral cleavage capabilities where a crRNA-target-binding activated Cas can randomly cut adjacent non-target single-stranded nucleic acids. Cas13 and Cas12a have been conveyed in the development of the SHERLOCK (Specific High-sensitivity Enzymatic Reporter UnLOCKing) and DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) systems for the detection of highly sensitive nucleic acid by combining with recombinase polymerase amplification (RPA) pre-amplification. Beyond RPA, several DNA sensors using CRISPR/Cas employed LAMP and PCR techniques, such as HOLMESv2, which uses the CRISPR/Cas12b-assisted one-hour low cost multifunctional highly efficient system. However, these CRISPR/Cas-based detection techniques generally involve distinct nucleic acid pre-amplification as well as additional manual processes, which unquestionably complicate the process and lead to contamination (Ding et al., n.d.).

### 6.1 HIV Detection

One of the world's leading public health concerns is HIV-1 infection, which affects more than 100 million people globally. For the rapid, ultrasensitive, selective, and visible identification of nucleic acid, an "AIOD-CRISPR" (All-In-One Dual CRISPR-Cas12a) test has been developed. To initiate extremely effective CRISPR-based nucleic acid identification, dual crRNAs are employed. Because all elements for nucleic acid amplification and for the identification of CRISPR are

completely combined in one reaction system, they may be incubated at a single temperature (37 °C), avoiding the requirement for individual pre-amplification and amplified product transfers. The AIOD-CRISPR test was developed to identify the human immunodeficiency virus type 1 (HIV-1). Because SARS-CoV-2 and HIV-1 are retroviruses, the AIOD-CRISPR assay's effectiveness is measured by identifying both DNA and RNA. Moreover, the AIOD-CRISPR assay's test findings, in particular, may be seen with the naked eye. As a result, it is believed that the AIOD-CRISPR assay will accelerate the development of next-generation molecular diagnosis based on CRISPR for using at the point-of-care purposes (Ding et al., n.d.).

Early detection of HIV-1 infection with inexpensive, quick point-of-care testing (POCT) is critical for establishing effective management of the HIV-1 epidemic. In this work, a real-time isothermal reverse-transcription recombinase-aided amplification (rRT-RAA) and CRISPR Cas12a-mediated HIV-1 detection test was generated and assessed. By employing a blue-light imager, the findings may be inspected by anybody, making it a feasible option for on-site testing. According to early results, the test may identify 20 copies of pure HIV-1 DNA or RNA per response or as few as 123 copies/ml of HIV-1 viral load in clinical collections. As tested on 155 samples collected with or without HIV-1 infection, rRT- RAA's sensitivity as well as specificity were 98.95% (94/95) and 100% (60/60). For example, it was 0.9865 in the case of the Chinese FDA authorized HIV-1 real time reverse transcription polymerase chain reaction (RT-qPCR) test. Furthermore, in China the recently advanced HIV-1 rRT-RAA assay could identify the main HIV-1 genotypes CRF01 AE, CRF07 BC, CRF08 BC, CRF08 BC, and subtype B. The first findings suggest that the rRT-RAA test, in conjunction with CRISPR Cas12a-mediated detection, might be a quick, easy, and reliable way to detect HIV-1 (J et al., 2021)

## 6.2 SARS-CoV-2 RNA Detection

CRISPR has gained popularity in the area of *in vitro* diagnosis in recent years. The specificity of its allele is crucial for its effective implementation in the development of high-precision therapy and diagnostics. Several new diagnostics based on CRISPR systems have been developed, including SHERLOCK, Cas13-based Rugged Equitable Scalable Testing (CREST), DETECTR, All-In-One Dual CRISPR-Cas12a (AIOD-CRISPR), Cas3-Operated Nucleic Acid detection and CRISPR/Cas12a-based-detection with naked eye readout (CRISPR/Cas12a-NER) (CONAN). The sample collection and detection process of SARS-CoV-2 have been depicted in Figure 4.

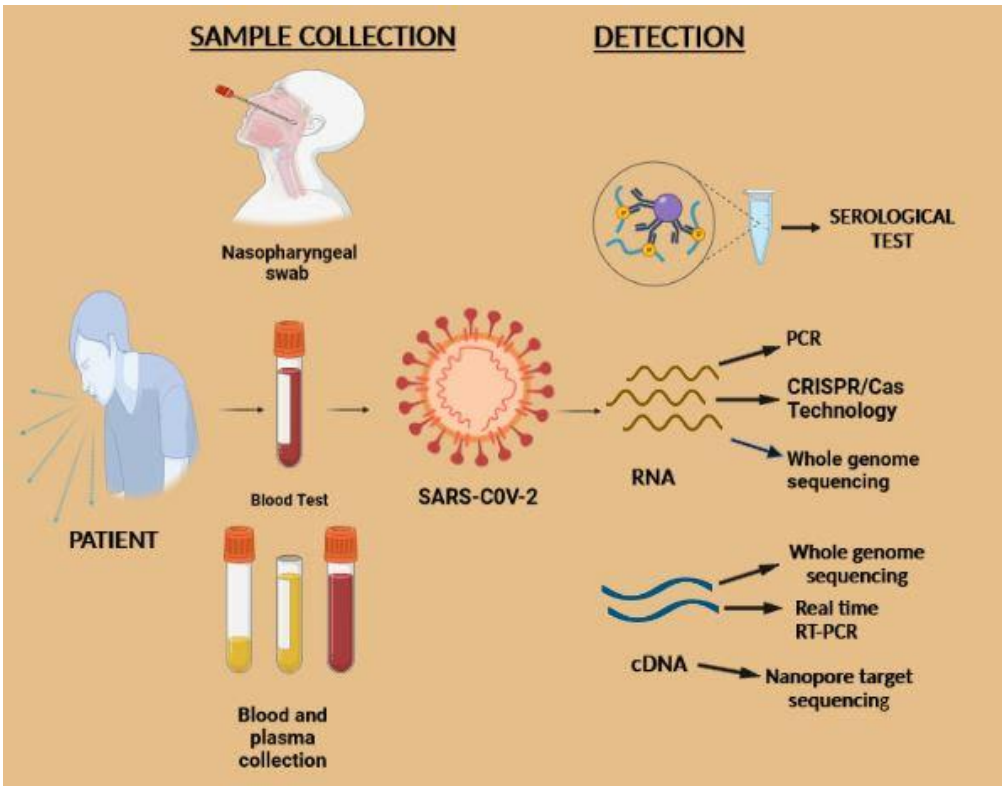


*Figure 4: Sample collection and detection process of SARS-CoV-2. It is possible to detect through using antibodies, RNA, cDNA from the sample. Different methods known as serological test, PCR, RT-PCR, genome sequencing, CRISPR/Cas technology are utilized for the identification of SARS-Cov-2 (Adapted from* Udugama et al., 2020).

### 6.2.1 SHERLOCK

SHERLOCK has recently been involved in the identification of COVID-19. In less than an hour, RNA extraction from patient samples may be used to start the detection process, which can then be read using a lateral flow method. The susceptibility of particular target sequences of COVID-19 utilizing the SHERLOCK technique is estimated to be between 10 and 100 copies per microliter of direct input (20 and 200 aM). Two genes, including the S gene and the Orf1ab gene, were targeted for detection of COVID-19 RNA in specimen nucleic acid extraction methods. Primer sequences and CRISPR gRNAs were utilized for the detection of RNA involving COVID-19 in specimens with the fewest off-target sequences from other human respiratory virus genomes, resulting in a highly specific assay. STOP (SHERLOCK Testing in One Pot) was recently established as a basic and convenient test chemical for the diagnosis of SARS-CoV-2 in one hour that is appropriate for point-of-care usage. STOPCovid's sensitivity is equivalent to that of RT-qPCR-based SARS-CoV-2 assays, as well as the LOD (limit of detection) is one hundred copies of viral genome per microliter (F. Zhang et al., 2020)

### 6.2.2 CREST

A CREST (Cas 13-based rugged, equitable, scalable testing) platform employing the detection of Cas13 paired with a heat cycling amplification phase (PCR), a linear amplification process (transcription) as well as enzymatic signal amplification through fluorescence identification was designed in order to construct a less expensive, highly delicate, and easy-to-handle detection technique of SARS-CoV-2. To overcome the requirement for costly equipment, CREST utilizes low-cost, bluetooth-enabled, battery-powered, field-ready thermos-cyclers (e.g. DIY Bio and miniPCR mini16). These thermos-cyclers use inexpensive methods to allow viral particle

multiplication and to make COVID-19 testing more accessible. The P51 cardboard fluorescence visualizer (powered by a 9 V battery) has been utilized as a flexible and simple to interpret device for visualizing favorable outcomes. The CREST technique has a limit of detection up to ten copies of a specific target RNA molecule per microliter, indicating that CREST is as robust as RT-qPCR. Besides, CREST equipment is expected to be 30–50 times less expensive than RT-qPCR, as well as more inexpensive and available than a variety of Cas13 dependent techniques that involve RT-Recombinase Polymerase (Rauch et al., 2021).

### 6.2.3 DETECTR

A quick (30 minutes), minimal, and efficient CRISPR-Cas12 lateral flow test for the identification of SARS-CoV-2 using RNA samples from respiratory swab was developed as part of the SARS-CoV-2 DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) project. SARS-CoV-2 DETECTR, a CRISPR-Cas12 derived assay for identification of SARS-CoV-2 from extracted RNA of patient specimen in less than 30 minutes, was developed and validated. This test conducts concurrent reverse transcription and isothermal amplification utilising loop-mediated amplification (RT-LAMP) from RNA isolated from swabs of nasopharyngeal or oropharyngeal in universal transport media (UTM), following Cas12 detection of preset coronavirus sequences, and through the split of a reporter molecule to validate virus identification. This technique has several benefits over conventional approaches such as qRT-PCR, including (1) isothermal signal amplification for fast target identification, which eliminates the requirement for thermocycling, (2) Target specificity: single nucleotide (guide RNAs at the N2 site (amplicon) of the nucleoprotein (N) genes of the virus may differentiate SARS-CoV-2 from SARS-CoV and MERS-CoV), (3) integration with portable, inexpensive reporting formats including such lateral flow strips, and (4)

A rapid growth process to handle emergent risks from new zoonotic virus (SARS-CoV-2 takes two weeks (Broughton et al., 2020).

### 6.2.4 AIOD

Nucleic acid may be detected using the AIOD-CRISPR (All-In-One Dual CRISPR-Cas12a) test, which is quick, accurate, specific, and visible. In this assay, chemicals used for the amplification of nucleic acid as well as identification through CRISPR are completely combined in a particular tube for reaction and incubated at 37 °C at a unique temperature because of the use of isothermal nucleic acid amplification methods including such LAMP 8 and RPA. Moreover, single pot reaction systems reduce work by eliminating pre-amplification and amplified product transfer. Furthermore, ssDNA-FQ reporters have been introduced to the AIOD-CRISPR assay to enhance detecting signals. Real-time detection employing fluorescence and color transformation of reaction solutions leads to the use of an AIOD-CRISPR assay as a point-of-care diagnosis. When Avian Myeloblastosis Virus (AMV) reverse transcriptase is combined with AIOD-CRISPR assay reagent, a one-step RT-AIOD-CRISPR is developed for the detection of RNA targets including such HIV-1 and SARSCoV-2 RNAs. RT-AIOD-CRISPR allows for the identification of RNA and doesn’t require the necessity for cDNA preparation. Besides, AIOD-CRISPR has been engineered to identify HIV-1 as well as SARS-CoV-2 without having the requirement for pre-amplification. In 40 minutes of incubation, the LOD of AIOD-CRISPR is expected to be low for example, in case of targeting RNA: 4.6 copies and for targeting DNA:1.2 copies. Furthermore, identification of HIV-1 with very minimal background compared to report real-time RPA confirms AIOD-CRISPR's ultra-specificity (Safari et al., 2020).

### 6.2.5 CRISPR/Cas12a-NER

To improve the COVID-19 diagnostic technique, CRISPR/Cas12a-NER (Naked eye readout) is established which is a CRISPR/Cas12a-based assay with visual interpretation. The assay shows significant accuracy with the qPCR assay, also identifies as low as 10 copies of the infectious gene in 45 minutes without the requirement of any special instrument, making it an appropriate on-site diagnostic technique for a small hospital or community health center. CRISPR/Cas12a-NER, which includes the Cas12a protein, SARS-CoV-2 particular CRISPR RNAs (crRNAs), and a single-stranded DNA (ssDNA) reporter, was designed to accurately and swiftly identify SARS-CoV-2 nucleic acid. An ssDNA sensor tagged with a quenched green fluorescent molecule was incorporated to facilitate on-site diagnosis, which will be fragmented by Cas12a because there is nucleic acid of SARS-CoV-2 in the detection technique, and under 485 nm light, the resultant green fluorescence can be observed with the naked eye (X. Wang et al., 2020)

### 6.2.6 Cas3-Operated Nucleic Acid detection (CONAN)

Cas3 protein, a part of the type I-E CRISPR-Cas system originated from *Escherichia coli* and *Thermobifida fusca*, may also induce nonspecific, target activated cleavage of ssDNA in addition to Cas12 and Cas13 effectors. This intriguing characteristic, along with isothermal amplification techniques, results in a fast, accurate, and instrument-free SARS-CoV-2 point-of-care test (POCTs). RT-LAMP (at 62 °C for 30 minutes), generally proceeded by a 10-minute incubation with CONAN at 37 °C, particularly identified SARS-CoV-2 utilizing N1 and N2 crRNAs. CONAN-LAMP had a lower LOD (102 copies) than DETECTR-LAMP and the CDC qRT-PCR test for SARS-CoV-2 diagnosis (Yoshimi et al., 2020).

## 6.3 Hepatitis B Virus Detection

Untraceable or relatively low DNA of hepatitis B virus (HBV) as well as drug resistance mutations in individuals may enhance the probability of HBV infection or induce persistent viral replication as well as other clinical difficulties. PCR-CRISPR (or PCR-Cas13a) is a CRISPR-based technique for detecting HBV and treatment resistance that has been proven to be very sensitive and feasible. In order to detect HBV DNA and identify YMDD (tyrosine-methionine-aspartate-aspartate) variants, specific CRISPR RNAs (crRNAs) were developed. HBV DNA was identified in 312 serum samples for HBV detection utilizing quantification PCR (qPCR) and PCR-CRISPR. In addition, 424 serum samples for YMDD testing were found using qPCR, direct sequencing, and assay. HBV DNA and YMDD medication resistance mutations may be detected in one copy per experiment. For the early diagnosis of HBV infection, medication resistance monitoring, and treatment counselling, this technique has a lot of potential (S. Wang et al., 2021).

## 6.4 Human Papillomavirus Detection

The human papillomavirus (HPV) is a dsDNA virus that causes malignancies of the cervix, anal and other organs. Clinical detection of HPV is now routinely used in cervical cancer detection and regular health examinations, utilizing a variety of PCR-based methods. A recent study provides a novel technique for identifying and characterizing targeted DNAs based on CRISPR, dubbed ctPCR3.0, which stands for CRISPR- or Cas9/sgRNA-typing PCR, version 3.0. This method identifies DNA which is targeted in a single homogenous phase: quantitative PCR (qPCR), which amplifies Cas9/sgRNA-broken DNA samples. Direct introduction of Cas9 and sgRNA into qPCR reaction and further isothermal incubation prior to qPCR program allow for homogenous detection of the target DNA within 2 hours. This new detection method, known as ctPCR3.0, does not need

exposing the detection tube at any point throughout the diagnostic procedure. The approach was thoroughly validated by identifying cloned HPV L1 genes from ten high-risk HPV subtypes. Two of the greatest HPVs, HPV16 and HPV18, were effectively identified in the DNA template of two HPV-positive cervical cancer cells, SiHa and HeLa using the L1 and E6-E7 genes of those viruses. Eventually, the ctPCR3.0 technique was proven to identify HPVs in a variety of clinical samples. It delivers a novel CRISPR-dependent DNA detection along with typing system, as well as a ready-to-use HPV clinical detection approach, by conducting these detection methods. The platform offers a wide range of applications in clinical diagnostics (B. Zhang et al., 2018).

# Chapter 7

# CRISPR-Cas System Based Antiviral Mechanism

## 7.1 HIV

### 7.1.1 Inactivation and Destruction of HIV-1 Provirus

Antiretroviral treatment (ART) is now being utilized to manage HIV infection at both the individual and global levels. As a result, both the number of new HIV infections and HIV death rates have been dropping steadily. The CRISPR/Cas9 system, which uses a guide RNA strand to detect and modify target DNA, is a recent advancement in genome editing that now provides a distinct method to inactivate HIV DNA. This CRISPR/Cas9 system combines a DNA endonuclease termed Cas9 with an RNA component that identifies a particular DNA region using a 20-nucleotide guide sequence (gRNA). DNA cleavage by Cas9 results the breakage of double-stranded DNA and get repaired by non-homologous end joining (NHEJ), occurring insertions or reductions at the target locations. A study has been done on immortalized T lymphocytes known as JLat10.6 cell line, where a noticeable efficacy of the CRISPR/Cas9 has been observed in targeting and inactivating the proviral DNA of HIV-1. Ten target locations across the HIV-1 genome has been reported and one region in the second exon of Rev (named T10) is detected with the maximum level of Cas9 mutation. In another study, CRISPR/Cas9 successfully establish the deletion of CCR532 in pluripotent stem cells having 33% effectiveness in contrast to 14% effectiveness of TALENS technique. As CRISPR/Cas9 complex attacks both inert transcription and active transcription of HIV-1 DNA with equal potency, studies indicate it would be effective and efficient in eradicating HIV-1 reservoirs (Zhu et al., 2015).

### 7.1.2 Disruption of Co-receptors of HIV

HIV-1 infects cells expressing CD4 as well as the co-receptors CCR5 or CXCR4. Although the CD4, CCR5, CXCR4 axis is recognized as the conventional way of HIV-1 entrance, there is a strong body of evidence indicating HIV-1 uses a variety of mechanisms to enter targeted cells. Moreover, HIV-1 infects CD4+ T cells along with macrophages. It has been demonstrated that macrophages may get infected by HIV-1 in a CD4 independent manner, resulting in viral endocytosis. Furthermore, investigations have demonstrated a CD4 and CCR5 or CXCR4 independent mechanism for HIV-1 infection of macrophages. This mode of entrance has been primarily attributed to macrophages' phagocytic character. Here, CRISPR gene editing technique was utilized to modify CCR5 and CXCR4 (Doudna & Charpentier, 2014).When the HIV-1 glycoprotein gp120 binds to CD4, it initiates a sequence of conformational alterations that enable this to one of two co-receptors (CCR5 or CXCR4). T-cells often die within 24–48 hours as a result of this. By modifying the Cas9 or ZFN methods, endonuclease enzymes may be transduced into targeted tissue after being packed into viral carriers. These target cells can be made immune to viruses that employ CCR5 or CXCR4 after effective genome editing. CCR5-using virus (R5) is represented by grey viral particles, whereas CXCR4-using virus is represented by red viral particles (X4). CRISPR/Cas9 can be utilized to eliminate the expression of HIV-1 co-receptors (Figure 5). A lentiviral vector has been used to transfer CRISPR/Cas9 to CD4+ T cells *in vitro*. The modified cells demonstrated resistance to CCR5-using viruses as well as a particular benefit over wild-type cells. Several researches have also found that inhibiting CXCR4 expression leads to an improvement in HIV-1 resistance *in vitro*. A recent survey showed the effective *in vitro* elimination of both the CCR5 and CXCR4 co-receptors. In 9% of transformed GHOST CXCR4+ CCR5+ cells, editing through CRISPR/Cas9 system resulted in bi-allelic loss of both co-receptors.

Considering the possible characteristics of gene editing in general terms, and CRISPR/Cas9 in specific, there are still difficulties. CRISPR/Cas9 has exhibited tremendous potential. However, it lacks the extensive validation of animal model observed with other genome - editing approaches like ZFNs (Allen et al., 2018a).

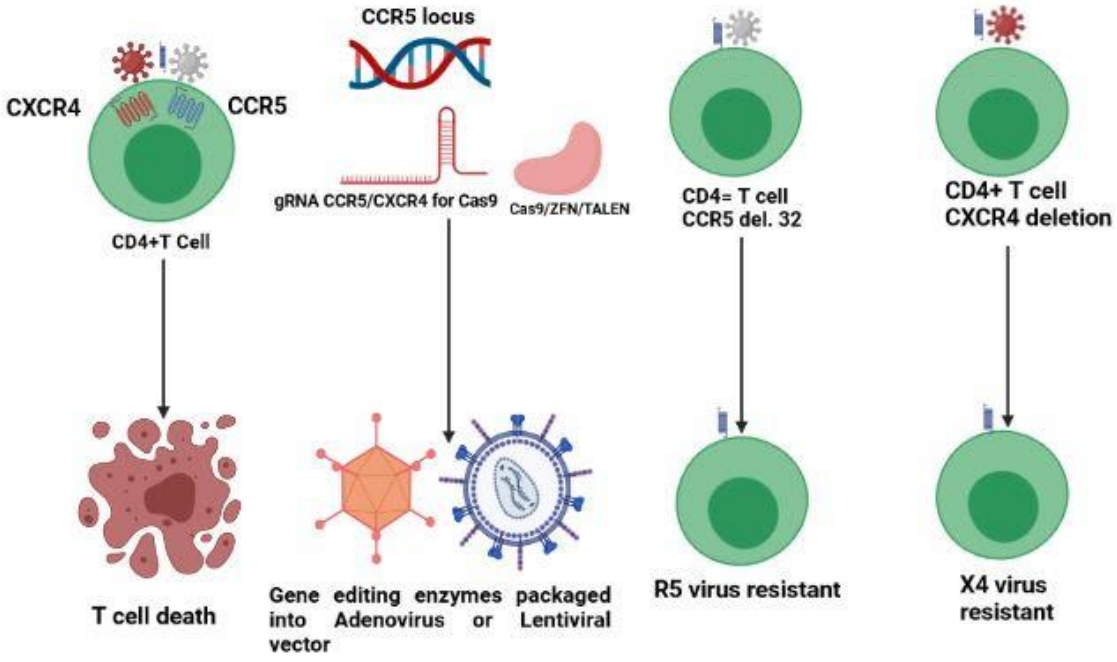


*Figure 5: Disruption of co-receptors of HIV-1. For the disruption of the expression of HIV-1 co-receptors CCR5/CXCR4, lentivirus or adenovirus vector has been used to transduce CD4+ T cells with CRISPR/Cas9 system (Adapted from* Allen et al., 2018b).

## 7.2 SARS-CoV-2

### 7.2.1 PAC-MAN strategy

CRISPR-Cas13 has developed as a highly potent antiviral strategy that uses sequence-specific crRNAs to defend that particular bacterial cells of host against the infection of bacteriophage. The RNA-guided endonuclease action of Cas13d is repurposed against viral infections like SARS-

CoV-2 in human cells, using a prophylactic antiviral human cell (PAC-MAN) approach. In lung epithelial cells, the Cas13d system can efficiently identify and break the RNA sequences of SARS-CoV-2 regions using correctly designed crRNAs. Furthermore, bioinformatics analysis revealed that minimum pools of six crRNAs may target 91 percent of the 3,051 sequenced coronaviruses. SARS-CoV-2 has been shown to have highly conserved portions of the viral genome that can be targeted using Cas13d. These genomic areas encode the RNA-dependent RNA polymerase (RdRP) and nucleocapsid proteins, both are required for coronavirus replication and activity in the context of SARS-CoV-2. RNA-dependent RNA polymerase (RdRP) catalyzes the synthesis of all viral mRNAs along with nucleocapsid binds to genomic RNA, shielding it and acting as one of the two major fundamental proteins in virions. Targeted suppression of these proteins might have a large impact on preventing virus generation and activity, as well as lowering viral burden through viral genome destruction. It should be highlighted that PAC-MAN is presently a proof-of-concept antiviral approach for effectively and widely targeting recurrent viral sequences through Cas13, and that several key stages must be completed before clinical trials may be conducted to potentially cure COVID-19. The efficacy and selectivity of crRNAs in order to inhibit the infection of cells in the respiratory tract with live SARS-CoV-2 virus will be validated as an immediately following step. While the PAC-MAN system is being studied as a prophylactic system that is delivered before viral attack, it is believed that after infection PAC-MAN might be utilized to decrease the level of viral load, which will be investigated. In near future, testing the selection method of crRNA might be coupled with recently reported algorithms that estimate crRNA efficacy to further expedite the validation procedure of crRNA. The crRNAs chosen for therapeutic testing will require to be checked for off-target effects through experiment, such as utilizing whole transcriptome RNA sequencing. Furthermore, the PAC-MAN system may block viral sequences *in vitro*, therapeutic

application would necessitate a reliable *in vivo* delivery mechanism into human respiratory tract cells. Lastly, PAC-MAN will be verified in appropriate models of preclinical trials such as ferrets or rhesus macaques, as well as primary human lung/airway organoid models, to assess its antiviral effectiveness and selectivity. Also, animal models will provide data on Cas13d-induced immunogenicity, a problem that has emerged in CRISPR-Cas9 research. As a result, the CRISPR-Cas13d system might be utilised to combat emerging viral threats (Abbott et al., 2020; Chan et al., 2020)

### 7.2.1 ABACAS

CRISPR enzymes of type V and VI both are RNA-programmable and target DNA and RNA effectors capable of knocking out specific genes. Crispr proteins like Cas12 and Cas13 both are effective in diagnosing and fighting single-stranded RNA (ssRNA) viruses. The capacity of these proteins to be programmed provides the path for the identification and destruction of RNA viruses through directly targeting RNAs that are similar to their CRISPR RNA (crRNA). The CRISPR elements can be linked to one of the key viral structural proteins including S protein, to allow Cas13 for entering into infected cells and selectively target the particular SARS-CoV-2 viruses. The integration of Cas13 to the particular antibody of SARS-CoV-2 S protein resulted in the creation of ABACAS (Antibody and Cas fusion), a potential antiviral treatment (Safari et al., 2020).Due to obstacles in the monoclonal antibodies advances, such as high expense of production and possible resistance mechanisms, studies have been devoted to employ biologics that can link to the S protein as an alternate to allow Cas13 and viral particles to be released selectively (Lei et al., 2020; Nalawansha & Samarasinghe, 2020a; Pang et al., 2020).

The combined form of ABACAS and SARS-CoV-2 has been illustrated in Figure 6. When viral infection attacks a cellular host by endocytosis, then ABACAS enters cell that is infected including the virus. The activation of ABACAS stimulated the Cas13 component, which recognizes and facilitates the destruction of viral RNA. Furthermore, ABACAS prevent viral entrance into target cells by suppressing the virus's protein. Peptidase Domain of ACE2 and Cas13 (PDCas13) is a current replacement for ABACAS in which the antibody component of ABACAS is substituted with the peptidase domain of ACE2 or a simple peptide of ACE2 (possibly attaches to SARS-CoV-2 S protein). PDCas13, on the other hand, is ineffective to cover all S proteins of SARS-CoV-2, yet co-delivery of PDCas13 along with virus particles to infectious cells activates PDCas13's antiviral action through RNA virus breakdown. Notably, among the FDA-authorized specific monoclonal antibodies targeting the S protein of SARSCoV-2. Also, ABACAS comprising SARS-CoV-1 nAb and PDCas13 including rhACE2 or ACE2 peptide are important accomplishments for evaluating specific and effective release of the CRISPR system to infected tissues and functioning as an antiviral agent against SARS-CoV-2 (Safari et al., 2020).

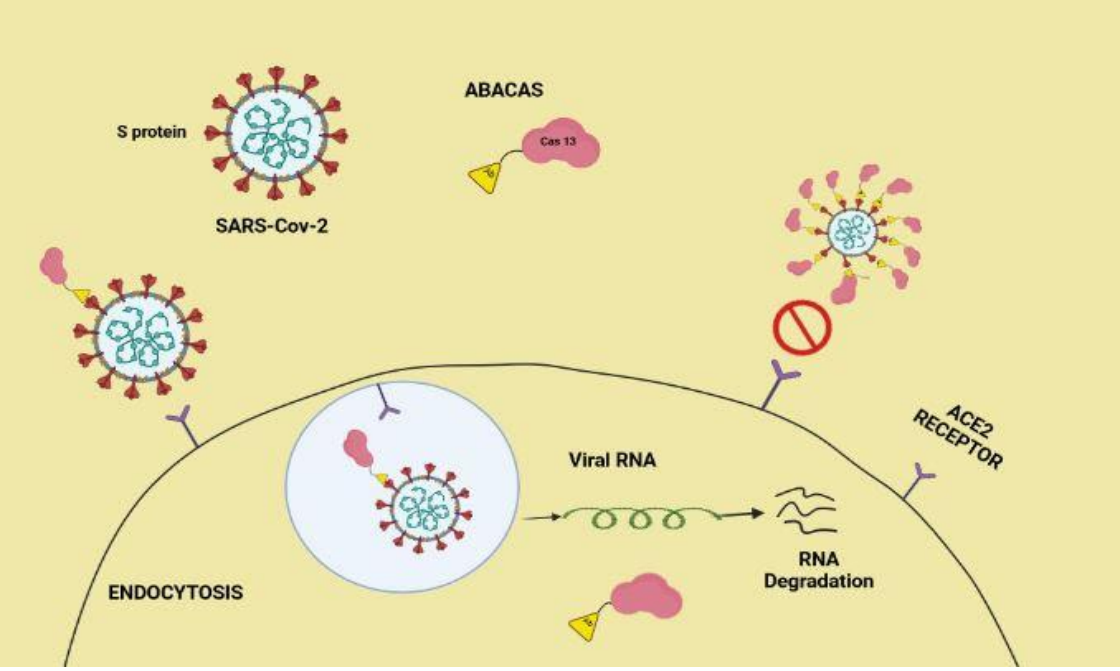


*Figure 6: Illustration of the bound form of ABACAS and SARS-CoV-2. Activation of Cas13 due to ABACAS leads to degradation of viral RNA (Adapted from Nalawansha & Samarasinghe, 2020b).*

## 7.3 Hepatitis B Virus

Hepatitis B virus (HBV) infection continues to be a worldwide health concern because chronic HBV infection can cause liver cirrhosis or malignancy. Recent antiviral treatments based on nucleoside analogues can suppress HBV replication however, do not break the covalently bound circular DNA (cccDNA) of HBV. Furthermore, because HBV is a retrovirus, it is more prone to produce mutations in reverse transcription, increasing the likelihood of evading standard antiviral medication therapy. Because HBV cccDNA has remarkable stability and decreases gradually with antiviral therapy, effective and non-toxic cccDNA elimination is a significant aim for HBV therapy (Werle-Lapostolle et al., 2004).The recently established CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) system is a potent tool for targeting cellular genomic DNA for gene editing. In this process, eight guide RNAs (gRNAs) were developed that targeted

particularly the conserved regions of distinct HBV genotypes and could effectively suppress the replication of HBV both *in vitro* and *in vivo* to assess the feasibility of employing the CRISPR/Cas9 system to damage the HBV DNA templates. As a result, this genome editing method has the potential to help avoid medication resistance induced by changes during viral replication (X. Liu et al., 2015).

## 7.4 Herpes Simplex Virus

CRISPR-Cas9 system has been utilized to edit many eukaryotic genes as well as different studies have been conducted to investigate its antiviral ability. The capacity of CRISPR/Cas9 to inhibit the replication of HSV-1 by targeting particular DNA regions important to viral protein production throughout initial and last stages of viral infection/reactivation was investigated to determine its translational capability for use to treat and eradicate HSV-1 infection. A specified set of sequentially produced viral proteins directs a persistent infection cycle during initial HSV-1 infection. Following viral entrance into cells, viral capsid proteins are discharged into the cytoplasm, inhibiting the synthesis of host protein. During the initial stages of viral infection, 100–200 copies of the infected cell protein 0 (ICP0), which is linked with capsid proteins, are carried to the internal tegument, facilitating nuclease-mediated entrance of capsid proteins in the region of viral replication. Simultaneously, the viral gene producing ICP0 is activated, also freshly manufactured ICP0 supports effective lytic infection development, which is more comparable to real-life infection than the models of high dosage infection. As a result, during low-dose viral infection, ICP0 promotes more efficiently to viral survival and proliferation. To evaluate their capacity to inhibit viral gene expression, the selection of the ICP0 DNA sequences was done and guide RNAs were produced to interact with Cas9. This technique was discovered to generate

precise indel mutations in the ICP0 sequence, generated no off-target effects or damage to host cells as well as prevented antiviral PML bodies from the breakdown by ICP0, demonstrating the possibilities of this model approach for creating anti-HSV-1 treatments capable of eradicating latent infection. According to research, our gene editing technique of creating a single nucleotide variation in the ICP0 coding region is extremely efficient in preventing viral replication. As a result, it presents additional evidence that CRISPR/Cas9 can be established as a novel therapeutic technique for HSV-1 infection as well as a prospective and long-term approach for removing HSV-1 DNA from latently infected cells (Roehm et al., 2016).

## 7.5 Human Papillomavirus

Cervical cancers in women are caused by high-risk HPV infections. Despite the effectiveness of many types of preventive vaccinations against HPV infection, there are currently no HPV-specific antiviral medicines available. Seven studies reported effective CRISPR/Cas9 anti-HPV applications that disrupted the HPV genome directly. Although Cas9 from Streptococcus pyogenes was utilized in the majority of the investigations, one used a smaller form of Cas9 from *Staphylococcus aureus*. The majority of the experiments employed lipofectamine-based transfection to introduce CRISPR/Cas9, whereas two utilized lentiviral or adenoviral transduction. Because of their biological relevance as viral oncogenes, two distinct sections of an HPV genome, E6 and E7, were selected for the synthesis of a panel of gRNAs. HPV DNA and mRNA, as well as viral proteins such as E6 and E7, were measured for CRISPR/Cas9-mediated cleavage effectiveness screening. Some research found a reduction in E6 or E7 mRNA or protein levels after using the CRISPR/Cas9 technology. The reactivation of tumor suppressor genes including p53, retinoblastoma protein (pRb), and p21 further revealed the inactivity of E6 and E7 genes. As

a result, cell or tumor growth suppression was also observed in all CRISPR/Cas9-targeted HPV investigations. Furthermore, one research found no off-target cleavage at top-ranked possible CRISPR/Cas9 recognition regions. Depending on these findings, CRISPR-Cas9-mediated HPV genome destruction can be considered one of the most efficient antiviral methods against HPV infection (Lee, 2019).

# Chapter 8

# Ongoing Clinical Trials and Approval of CRISPR Technology

## 8.1 Trials in Mice to Target HSV-1

Herpes simplex virus (HSV-1) is one of the prominent reasons for infectious blindness. HSV-1 infection and latent reservoirs in the trigeminal ganglia are not completely eradicated by existing therapies. The therapeutic possibility of CRISPR on infectious disease is intriguing, and it exhibits viral elimination in underlying infectious reservoirs in HIV-1 infected humanized mice by integrating antiviral prodrugs with CRISPR. HSV-1 genomes are therefore specifically targeted utilizing mRNA-containing lentiviral vectors that concurrently release SpCas9 mRNA and viral gene-targeting gRNAs (also known as HSV-1-erasing lentiviral particles, or HELP) (Yin et al., 2021). An AAV-based HSV-1 endonuclease was delivered in a mouse model of latent HSV infection, nevertheless neither a noticeable depletion of viral genome nor therapeutic effectiveness was seen (Aubert et al., 2016). Recently the fact has been established that a detectable eradication of latent genomes and treatment effectiveness was observed utilizing an enhanced AAV vector and dual-mega nuclease specifically targeting the HSV genome. Thus far, CRISPR's anti-HSV action has only been studied *in vitro*, and no research has demonstrated CRISPR's therapeutic effectiveness against HSK *in vivo* (Oh et al., 2019; van Diemen et al., 2016).

## 8.2 Trials of Combined Antiviral Therapy with CRISPR Gene Editing for HIV

The removal and eradication of properly incorporated proviral DNA from affected cells and tissues is required for HIV-1 elimination. When administered in a clinical setting, antiretroviral treatment

(ART) prevents viral infection by halting several stages of the viral life cycle. Although it has been shown to be effective, antiretroviral therapy (ART) is unable to eradicate integrated versions of HIV-1 proviral DNA in the host genome. This means that the virus survives latently in infectious reservoirs and ceasing ART induces rapid viral reactivation as well as the development of acquired immunodeficiency syndrome (AIDS). To meet this demand and provide an appropriate treatment approach, our laboratories develop extremely hydrophobic lipophilic viral reservoir capable of tackling antiretroviral prodrugs known as long-acting slow-effective release ART (LASER ART) (Edagwa et al., 2018). Regardless of how effective the medicines are at limiting viral infection, LASER ART alone is not capable enough to clear the afflicted host of latent HIV-1. As a result, we are developing CRISPR Cas9 system depending genome editing technique employing AAV delivery that eradicate segments of integrating HIV-1 pro-viral DNA from the host genome precisely and effectively(Hu et al., 2014). As a result, the two methods are coupled to see if LASER ART and CRISPR-Cas9 therapies can work together to eliminate viruses. A research model of human infectious disease has been shown to be able to eliminate HIV-1 that is capable of reproduction. Viral clearance has been achieved in HIV-1-infected spleen and lymphatic tissues, humanized mice with previously infected solid organs cured with laser ART and AAV-CRISPR-Cas9. Thus, viral eradication can be achieved using an unique combination of LASER ART and a gene editing approach (Dash et al., 2019).

## 8.3 FDA's Emergency Use Authorization of CRISPR SARS-CoV-2 Kit

The development and fast progression of SARS-CoV-2 around the globe has demanded awareness and action in both public health laboratories and health-care services along with other aspects of community broadly. Specific and sensitive tests for viral detection are essential for correct

identification of patients, evaluation of the severity of the spread, tracking of intervention measures, and surveillance studies, among other things (Sherlock Biosciences, 2020). As one example, SHERLOCK- a CRISPR-based diagnostic tool also can be referred to as (Specific High-sensitivity Enzymatic Reporter unLOCKing). Health practitioners use the Sherlock™ CRISPR SARS-CoV-2 kit in patients who are suspected to be infected with Covid-19. This kit is intended to identify RNA and for that the specimens from upper respiratory (including nasal swabs and oropharyngeal swabs) and lavage of Broncho-alveolar is collected. Clinical samples are treated with the PureLink™ Viral RNA/DNA Mini Kit to extract RNA. The Sherlock™ CRISPR SARS-CoV-2 kit consists of two stages. The first phase is reverse transcriptase loop-mediated amplification (RT-LAMP), which involves converting specifically targeted genomic RNA to DNA of SARS-CoV-2, which is then replicated by a strand-displacing DNA polymerase. Transcribing this amplified DNA activates CRISPR complexes, which are specifically designed to cut target RNA sequences. The breakage of nucleic acid reporters causes a fluorescence readout to be observed by a plate reader as a response of this collateral action (Sherlock Biosciences, 2020). However, their versatility and broad adoption are still restricted by the requirement for amplified products to be moved between tubes, raising the possibility of contamination and human error, and by result interpretation, which has only been done for lateral flow-based readouts (Arizti-Sanz et al., 2020). The Sherlock™ CRISPR SARS-CoV-2 kit is for certified professionals in the clinical laboratory who have been particularly educated and trained in non-automated molecular *in vitro* diagnosis processes. This kit is intended for use only under Food and Drug Administration's (FDA) Emergency Use Authorization (Sherlock Biosciences, 2020).

# Chapter 9

# Challenges and Future Directions

## 9.1 Challenges

The genome editing offers tremendous promise for improving human health but there are serious concerns connected with its misuse, whether deliberate, irresponsible, or unintentional. At the same time genome editing allows new breakthroughs and the development of new technologies to more precisely modify living organisms. These developments might be utilized to cause significant damage, whether intentionally via the development of a biological weapon, accidently in the case of a biosafety failure that leads in the release of an altered organism into the environment, or unintentionally through the discrepancy (Kirkpatrick et al., 2018). Scientists are enthusiastic about CRISPR-based treatments. However, the technology is still in its early stages, with the first attempts just recently reaching clinical trials. Although CRISPR has eased and expedited genetic research, clinical applications still need significant skill in the design, manufacturing, and distribution of therapeutic components. Therapies based on genome editing, like other high-tech medicines, will be expensive, creating obstacles to accessibility. Current regulatory procedures might potentially stifle progress. Clinical studies in the United States are expensive, and present regulatory frameworks do not permit universal authorization of a personalization-compatible technology like CRISPR editing. Aside from the fundamental technical problems involved with prospective CRISPR treatments, such as the inefficiencies of homologous repair and issues about off-target mutagenesis, the costs of manufacturing, testing, and distribution must be drastically lowered in order to make the advantages of genome editing available to those in greatest need (Wilson & Carroll, 2019).

## 9.2 Future Directions

The CRISPR-Cas technology has exhibited great potential in inhibiting the replication of viral DNA or RNA both *in vitro* and *in vivo* and as a robust viral genome diagnostic tool. However, it needs to be ensured that the viral DNA or RNA targeted by the Cas proteins do not match with that of the human DNA or mRNA transcript sequences, which would otherwise degrade the human genome. Moreover, one of the fundamental barriers encountered in the field of gene editing is the inadequacy of an efficient delivery system as the components of the CRISPR system are too large to be taken up by the target cells easily. This calls for the need of designing molecular tools which would effectively deliver the components and therefore, reduce off-target activity and increase therapeutic efficacy.

# Chapter 10

# Conclusion

To conclude, the life cycle of viruses is intensely linked to host cell metabolism, making their eradication a therapeutic challenge. Since the beginning, the use of small-molecule inhibitors of virus-specific enzymes as an antiviral treatment is the most effective. However, for the majority of latency-associated persistent viral infections, this traditional antiviral treatment has proven ineffective. In this regard, the emergence of technology like CRISPR-Cas system has an unparallel potential for direct targeting of a viral genome. Hence, it will lead to the development of a novel antiviral tool aimed at achieving a previously unimaginable, complete cure. Regardless of the fact that there are several obstacles that must first be overcome prior to this revolutionary CRISPR/Cas technology can be fully transitioned from a preclinical study to an effective antiviral treatment, the therapeutic potential of the antiviral CRISPR/Cas system offer constant impetus for the development of new treatments against viral infections. Undoubtedly, this novel antiviral method would allow chronically infected individuals to quit administering life-long medications in the near future.

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