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Antiviral research and detecting viral nucleic acids based on the CRISPR-Cas technology

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Abstract. Viruses constructed from nucleic acids, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), are encased in a protein covering and require a living cell to reproduce. Symptoms of viral infections can range from asymptomatic to severe illness, and their impact has become increasingly apparent in recent years. For instance, globally, the Corona Virus Disease 2019 (COVID-19) epidemic alone has resulted in millions of infections and millions of fatalities. Consequently, the development of effective tools for antiviral resistance is an urgent need. Early detection of viral infections can be achieved through nucleic acid detection, while gene editing techniques offer a promising solution for treating and counteracting the harmful effects of viruses, thereby minimizing economic and property loss. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and associated (CRISPR-associated, Cas) protein system (CRISPR-Cas) is an emerging gene editing tool that has demonstrated remarkable accuracy and efficiency with its rapidly expanding applications. This review delves into the principles and applications of Cas12a, Cas13, and other systems in nucleic acid detection, highlighting the significant contributions of Cas9, Cas12a, Cas13, and other systems in establishing resistance against DNA and/or RNA viral infections, and outlines the strengths and limitations of the CRISPR-Cas system in current applications. Precautions and suggestions for the system's safe application are also presented, with an optimistic outlook for its prospects.

Keywords: CRISPR-Cas, virus, viral nucleic acid detection.

1. Introduction

Viruses are small, uncomplicated organisms that lack cells and have a single kind of nucleic acid, either RNA or DNA. They rely on living cells to parasitize and reproduce through replication, making them unique infectious agents. A virus lacks its enzymatic system and metabolic apparatus, being composed only of a strand of nucleic acids and a protein casing. Therefore, a virus can only replicate, transcribe, and translate within a host cell. Once inside the host cell, the virus can use its resources to complete its life cycle and create a new generation of viruses based on the genetic information found in its nucleic acid. While some viruses, such as phages and insect viruses, can be beneficial for treating bacterial infections and agricultural pests, respectively, many viruses can be harmful to human life, such as Human Immunodeficiency Virus (HIV) and rabies. Additionally, viruses such as influenza and hepatitis viruses can cause disease, while others like the Potato Virus Y (PVY) can damage property.

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Antiviral drugs are a class of medication used to treat viral infections, such as interferons and antibodies. Interferons are man-made replicas of natural interferons that can slow or halt viral replication, although they may have side effects[1]. Antibodies are primarily found in the blood of people who have recovered from viral infections (recovery serum) or in the laboratory from altered living cells that produce targeted antibodies (monoclonal antibodies). Unfortunately, many viral infections do not have effective antiviral treatments available. The way antiviral drugs work is by disrupting viral replication. However, viruses are very small and replicate inside cells, using the cell's metabolic functions. Because of this, antiviral drugs are only able to target a limited number of metabolic functions within the cell.

In contrast, bacteria are larger and typically replicate outside of cells, allowing antibiotics to target many different metabolic functions. This makes developing antiviral drugs more challenging than developing antibiotics. Additionally, while most antiviral medications are only effective against one or a few viruses, antibiotics are frequently effective against a wide variety of bacteria. Furthermore, antiviral drugs can have toxic effects on human cells, and viruses can become resistant to them.

To improve the effectiveness of viral infection treatments and minimize damage, scientists are exploring genetic engineering to detect viruses and advance prevention and treatment. The CRISPR-Cas methodology is frequently employed by bacteria to be a defense mechanism against foreign DNA incursions. This system employs short palindromic repeats, which use small RNAs to detect and neutralize invading DNA with specificity. There are two classes in the system, one for types I through IV (excluding II) and one for all other types. The system's specificity not only grants it powerful gene editing capabilities but also permits very sensitive identification of certain DNA sequences. As a result, Utilizing the CRISPR-Cas methodology, viral DNA may be found rapidly and selectively with high sensitivity. It can also be genetically engineered to create organisms that are resistant to DNA infection in plants and animals, making it effective against exogenous DNA. It's extremely probable that the unique and cutting-edge genome engineering tool, the CRISPR-Cas methodology, will make a significant contribution to the identification, avoidance, and management of viruses.

This review seeks a thorough rundown of the CRISPR-Cas system as well as its potential in human anti-virus efforts. It delves into the system's working principle, and application scenarios, as well as its pros and cons. By doing so, this review focused on shedding illumination on the development direction of the system, offering valuable insights and ideas for future researchers.

2. Mechanism of action of CRISPR-Cas

CRISPR is a short palindromic repetitive sequence found only in prokaryotic cells, i.e., bacteria and archaea, and genes associated with it are known as CRISPR-associated genes (cas genes). Three separate mechanistic steps make up the CRISPR-Cas immunization process: adaptation, expression, and interference.

During the first phase, bacteria integrate new spacer regions from viral genes upon phage attack, and the bacterial cellular resistance against bacterial infections is changed by the insertion or deletion of certain particular spacer regions [2]. Therefore, microbes have phage resistance due to the combination of CRISPR and Cas genes, and the specificity of the resistance depends on the similarity of the spacer-phage sequences. Cas proteins are also involved in the adaptive phase of prokaryotic cells, and the most conserved of them is the Cas1 protein [3]. Cas1 proteins with integrase activity are usually grouped in a synthetic complex with Cas2 proteins with endonuclease activity, which together catalyze the introduction of spacer regions [2]. A new spacer region is created when Cas1 enzymes cut a portion of an alien species' genome close to the Proto-spacer Adjacent Motif (PAM) and integrate it into the CRISPR locus. During this process, CRISPR RNA (crRNA) undergoes transcription and changes to become mature RNA [4]. In prokaryotic cells, the CRISPR motif is the region that preserves infected foreign genetic information.

During the expression phase, a DNA strand with a CRISPR site is transcribed into a messenger RNA (mRNA) called pre-crRNA. Numerous repeating CRISPR complementary sequences and alien species' genomic sequences can be found in pre-crRNA [3]. crRNAs are formed from pre-crRNAs, and their final compositions vary for different Cas types, which are classified into I, II, and III types. A repeated

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CRISPR genome and a foreign species' genome are present in the crRNA of process type I. The foreign genome sequence repeats do not create loops with repetition, but the CRISPR sequence does, and as a result, the crRNA is digested by the Cas6e and Cas6f nucleases. The trans-activating CRISPR RNA (tracrRNA) molecule plays a crucial role in process category II. Process type III involves the direct destruction of both the foreign species' genomic sequence and every repeat of the CRISPR sequence by the Cas6 nuclease [4].

During the interference phase, specially encoded crRNAs are integrated into Cas proteins as guide RNAs to form CRISPR-Cas complexes [4]. This complex uses information from the exogenous genome in the crRNA to recognize, detect, and inactivate it. As a result, the CRISPR-Cas methodology is commonly utilized within agriculture as well as industry to provide virus resistance to cash crops [5]. In addition, the system is a potent tool for genome research, allowing the editing of gene sequences in a variety of species using different vectors.

3. Utilizing the CRISPR-Cas system to identify viral nucleic acids

The increasing frequency of epidemics as well as attacks by disease-causing viruses has led to a growing need for quick, precise, and inexpensive nucleic acid detection techniques. Studies have demonstrated that DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR) [6], Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) [7], one-Hour Low-cost Multipurpose highly Efficient System (HOLMES) [8], and other assays may become excellent biomedical diagnostic tools.

3.1. DETECTR

The RNA-guided CRISPR-Cas12a (Cpf1) proteins are an integral component of the bacterial adaptive immune system, binding and cleaving DNA. Similar to CRISPR-Cas9, in genome editing, Cas12a appears to be used similarly due to the capacity resulting in specific breaks in double-stranded DNA (dsDNA). To be more precise, RNA-directed DNA binding triggers Cas12a's non-specific single-stranded DNA (ssDNA) cleavage function, which breaks down ssDNA molecules. In contrast, other Cas12 enzymes share this common feature of target-activated non-specific single-stranded deoxyribonuclease (ssDNase) cleavage [6].

DETECTR is a technique that Chen et al. developed by combining Cas12a-ssDNAase activation with isothermal amplification., which has the same sensitivity for DNA detection as for human papillomavirus (HPV) and other common viruses [6]. Amidst the COVID-19 pandemic, Broughton et al. utilized a method called loop-mediated amplification (RT-LAMP) to simultaneously amplify and transcribe RNA from nasopharyngeal or oropharyngeal swabs in universal transport medium (UTM) [9]. After this, particular coronavirus sequences were found using Cas12, and reporter molecules were then cleaved to confirm the presence of the virus [9]. The detection process utilized only a few necessary primers, detection reagents, and instruments [9]. It is evident that DETECTR currently serves an intriguing purpose in discovering epidemic infections quickly and effectively.

3.2. SHERLOCK

The RNA-directed ribonuclease (RNase) Cas13, which consists of four different family members (Cas13a-d), generates several cleavage locations in RNA targets' single-stranded regions based on particular base preferences. Additionally, it exhibits promiscuous RNase activity that is dependent on the target and causes bystander RNA molecules to be trans-cleaved, an effect that is referred to as side branching activity. Hence, the detection of nucleic acids with side branching activity can be achieved by using reporter molecules that are fluorescently labeled, antigenically labeled, etc., along with steps such as preamplification and the T7 RNA polymerase promoter introduction, i.e., specific and highly sensitive enzyme-mediated unlocking (SHERLOCK) [7].

Using the Cas13a-based molecular detection platform SHERLOCK, particular variants of the Zika viruses, dangerous microorganisms, human DNA genotyping, and changes in cell-free cancer DNA may all be detected [10]. Additionally, SHERLOCK reaction materials are easily reconstituted on paper for usage in the field after being stored for long-term preservation without a cold chain [10].

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3.3. HOLMES

SHERLOCK is very convenient for detecting target RNA, but since in vitro conversion from DNA to RNA needs to be performed prior to the experiment, it can be time-consuming to identify the sequences of DNA. This led to the development of the HOLMES.

Cas12a of the type 2 V-A system performs side branching cleavage of non-target ssDNA when forming a ternary combination of target DNA, crRNA, and cas12a [8]. Taking advantage of this property, HOLMES uses a bursting fluorescent ssDNA reporter as a probe and together alongside the Cas12a/crRNA twofold multifaceted DNA target forms a ternary configuration if it has been included within the response environment, which then trans-cut the non-targeted single-stranded DNA reporter, allowing the fluorophore to separate from the bursting agent and emit Hexachloro (HEX) fluorescein [8].

In 2019, a group of researchers from the Chinese Academy of Sciences and others [11] developed HOLMESv2. They developed it by using a V-B-type mechanism and thermophilic RNA-guided Cas12b. By merging target identification and nucleic acid enhancement into a single procedure, they also made the operational procedures simpler. This was done to prevent cross-contamination.

With this method, human single nucleotide polymorphisms (SNPs), DNA viruses, RNA viruses, etc. can be detected [8, 11]. In HOLMES and HOLMESv2, the nucleic acid may be detected quickly and cheaply by using PCR, which uses inexpensive chemicals instead.

3.4. Other detection methods

When utilizing Casase without target upstream pre-amplification, the majority of CRISPR-based diagnostics report a limit of detection (LOD) in the picomolar range [12]. This LOD only permits identification when the sample DNA or RNA concentration is sufficiently high, as is the case for COVID-19 at high concentrations early in the infection. Therefore, to increase the sensitivity, LwaCas13a can be used in conjunction with Csm6, a CRISPR III RNase. Since LwaCas13a has side branch cleavage activity, Cas13 can produce a Csm6 activator, which enables amplified signal detection [13].

Furthermore, methods such as pre-amplification and quantification of biomarker concentrations can be used to obtain the appropriate LOD for clinical utility.

4. The implementation associated with CRISPR-Cas platforms in addition to antiviral tactics

The CRISPR-Cas system, a mechanism exclusive to prokaryotes, has made a significant contribution to the protection of prokaryotes against external genetic material. As a result, people associate the system with the introduction of eukaryotes imparting immunity against relevant viruses, or with the employment of the system to directly or indirectly attack viruses in order to cure viral illnesses that are infectious. Most recently, with the continuous maturation of related technologies, this system has gradually become an antiviral tool with vast prospects, bringing gospel to the fight against viruses.

4.1. Establishment of virus resistance

4.1.1. Establishment of DNA virus resistance. Depending on the dependence of the virus on the host, the genome of influenza viruses may be targeted for destruction using the CRISPR-Cas methodology, and the use of this methodology can target the destruction of host factors to resist viral infection and inhibit viral life activities as well.

The type of CRISPR-Cas methodology that has been studied more extensively is type II. Two tiny RNAs, crRNA as well as tracrRNA, and a single guide RNA (sgRNA), a form of these two small RNAs that have been artificially combined, are employed to lead the Cas9 effector DNA endonuclease to DNA targets in this system [14]. The 20-base pair seed sequence and the 3-base pair PAM (5'-NGG-3') make up a common targeting location for the CRISPR-Cas9 methodology following the Watson and Crick requirements are met, that is, Cas9-mediated dsDNA breaks occur when the CRISPR-Cas9 target, as well as the Cas9-bound sgRNA, complement each other. The host DNA repair mechanism as well as

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one of the non-homologous end joining (NHEJ) processes often resolve this [14]. Random nucleotide insertions, deletions, and substitutions around DNA cleavage sites take place during the reparation of DNA caused by NHEJ. As a result, the viral genomic critical regions that code for proteins and/or cis-regulatory components are disrupted, which stops the virus from replicating and eventually causes the host cell to become antiviral.

Based on the principle of action of the type II system, Kennedy et al. [15] targeted and disrupted the E6 and E7 genes in SiHa and HeLa cells, fruitfully blocking their expression and accelerating apoptosis of infected cells, which in turn prevented virus transmission.

4.1.2. Establishment of RNA virus resistance. As the main types of plant viruses, for RNA viruses, Cas13a or FnCas9 is able to strike the genome of the virus, or Cas9 could be employed for targeting the host factors of RNA viruses.

By hindering the ssRNA phage's ability to be cleaved by MS2 in E. coli, Cas13a was shown to provide immunity to phage; in vivo and in vitro, it breaks down the intended ssRNAs with the direction of the control of a crRNA containing a 28-nucleotide spacer region sequence [16]. Zhang et al. [5] reprogrammed sgRNA-FnCas9 to produce cucumber mosaic virus (CMV)-resistant plants, also by Agrobacterium-mediated transformation.

Alternatively, targeting viral host factors using Cas9 is also a way to establish viral resistance. The eIF4E genome in cucumbers has been selected for editing using CRISPR-Cas9 by Chandrasekaran et al. [17], and they screened pure non-transgenic cucumbers with a knockout of the eIF4E gene, which had significant viral resistance through backcrosses.

4.2. Treatment of viral infections

Since the Hepatitis B Virus (HBV) represents a DNA virus, when it reaches the nucleus, its genome is covalently closed circular DNA (cccDNA). Reverse transcriptase inhibitors, such as nucleic acids and nucleotide analogs, are the mainstay of current HBV therapy. Lin et al. [18] designed eight gRNAs against type A HBV, which together with Cas9 protein significantly reduced HBV core antibody and the surface antibody to HBV in Huh7 cells containing HBV expression vectors, indicating that the CRISPR/Cas system can cut HBV-specific sequences in cell models. Subsequently, researchers further demonstrated in a mouse model, in which hepatocytes plasmid carrying the HBV nucleotide might be destroyed by CRISPR/Cas9, thereby removing the plasmid and leading to a decrease in the level of HBV surface antibody within the blood.

Herpes viruses are a class of large DNA viruses with an envelope, which are best characterized by a latent cycle that allows them to under-express their proteins in cells thereby evading immune surveillance, and ultimately leading to lifelong infection because the host cell is unable to remove the virus from the infected cell. Herpes Simplex Virus type 1 (HSV-1) is one of the human neuroleptic α-herpesviruses, and Roehm et al. [19] effectively inhibited HSV-1 replication and reversed HSV-1-induced disassembly of pro-granulocytic leukemia nucleosomes through CRISPR-Cas9-based ICP0 targeted disruption.

Cervical cancer in women is brought on by high-risk Human Papillomavirus (HPV) infection, and although various prophylactic vaccines against HPV infection have been successfully developed, specific antiviral drugs against HPV are not yet available. Kennedy et al. [15] targeted and disrupted the E6 as well as E7 genes in SiHa et HeLa cells with HPV infection, accelerated apoptosis of infected cells, and then cleared HPV to block its transmission.

4.2.1. Treatment of RNA virus infections. The primary pathogen responsible for Acquired Immune Deficiency Syndrome (AIDS) is HIV Type-1 (HIV-1), and although the advent of highly active antiretroviral therapy (HAART) has changed AIDS from a deadly and untreatable to treatable and manageable, its lifelong persistence and incurability have become a huge burden for many AIDS patients. The drawback of HAART's inability to cure AIDS lies in its inability to remove HIV DNA integrated into chromosomes, and thus high hopes have been placed on the CRISPR-Cas9 method for eradicating

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the HIV genome. The CRISPR-Cas9 technique has been utilized by Ebina et al. [20], with the intention of altering the HIV-1 RNA as well as controlling how it is expressed. After transfecting the CRISPR-Cas9 system targeting long terminal repeat (LTR) sequences into LTR-expressing dormant and induced T-cells, they found that the LTR gene expression was significantly absent, confirming that CRISPR-Cas9 was able to effectively cleave and mutate LTR target sites, inhibiting the expression of activated proviruses and reactivation of latent viral reservoirs. It was also found that this technique could eliminate internal viral genes from chromosomes in host cells. This technique has been employed by Hou et al. [21], in order to select the human CXCR4 gene, a secondary receptor of HIV, to enable CD4+T cells to develop specific resistance to HIV, inhibit HIV-1 infection, and delay the course of the disease by knocking down the expression of the somatic CXCR4 gene.

Positive-stranded, ssRNA viruses include the Hepatitis C Virus (HCV). Many powerful direct antiviral drugs (DAA) that target the proteins that make up the virus have been developed, and as a result, most infections with HCV—regardless of genotype—can now be cured with the right pharmaceutical treatments. Drug-resistant HCV genotypes can be targeted by disrupting the HCV genome with CRISPR-Cas9 technology. Price et al. [22] transfected cells with gRNA-FnCas9 targeting the HCV genome, which significantly reduced the expression of HCV proteins and ultimately inhibited HCV replication.

5. Conclusions

CRISPR-Cas technology is quickly becoming a valuable tool for detecting nucleic acids not only in animal viruses but also in plant viruses and bacterial typing. Both Cas12a and Cas13 have shown impressive accuracy, made virus detection and monitored possible through DETECTR, SHERLOCK, HOLMES, and other methods. These methods are gaining popularity due to their efficiency, affordability, ease of use, and quick results. They hold great potential for contributing to the cost-effective and efficient detection of viruses.

Furthermore, the recently uncovered class V Cas12a exhibits a reduced molecular weight compared to Cas9 and a higher count of target sites. These factors make the CRISPR-Cas12a technology more proficient in precise gene editing than the CRISPR-Cas9 technology. It is evident that the CRISPR-Cas12a technology has a bright future in advancing antiviral research, and it is expected to become a potent weapon in the fight against viral infections.

Although CRISPR-Cas has shown promise in genetically modifying organisms and antiviral therapies, its cutting method can pose risks and unintended effects on their original genomes. To ensure safety, it is recommended to use Cas9nickase due to its reduced off-target properties. Additionally, numerous viral genomic locations might need to be concurrently targeted simultaneously to prevent drug-resistant mutations and inhibit viral escape. When selecting gRNAs for the CRISPR-Cas technology, it's crucial to prioritize the most critical and conserved viral sequences. Similarly, when targeting host factors to combat viruses, it's important to focus on factors that are necessary for the virus but not critical for the host. Finally, monitoring the host's post-targeting activities is crucial to avoid any unintended consequences.

The potential of CRISPR-Cas technology for virus detection and antiviral research is promising. However, its rapid emergence has presented various challenges and limitations that require careful attention. Nevertheless, with continuous refinement and improvement, it is reasonable to believe that these obstacles can be mitigated or even eliminated, resulting in a safer and more effective CRISPR-Cas tool. The benefits of this progress will be significant, as it will greatly contribute to the progress and success of antiviral research, prevention, and treatment, ultimately promoting the health and well-being of society.

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