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Role of Crisper CAS9 Technology in the Diagnosis of Covid19

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ABSTRACT

Covid 19 is a pandemic disease came in existence in 2019. It is also called as the Human Corona Virus disease which is caused by SARS-CoV-2. Currently more than 215 countries around the world are being reported to be the sufferers of this disease. When this disease came into existence the diagnosis of the disease became a major task for the scientists and doctors across the world. A method known as RT PCR (Reverse Transcriptase Polymerase Chain Reaction) was applied during that crucial period to detect this disease. Among other diagnostic tools, CRISPER (Clustered Regularly Interspaced Short Palindromic Repeats) Cas system is being investigated for rapid and specific diagnosis of COVID-19. CRISPER-Cas technology is a highly flexible RNA guided endonuclease (RGEN) based nucleic acid editing tool that has transformed the field of genomics, gene editing, gene therapy and genome imaging. As compared to RT PCR which gives the result of diagnosis in 4-8 hours, the CRISPER Cas based method diagnose it within an hour. So, this technique came out to be a very appropriate and less time-consuming method for detection of the disease. This article is focuses on explaining the potential use of CRISPER Cas9 based approaches for the development of accurate and rapid diagnostic technique and antiviral therapy for detecting and limiting the spread of COVID-19. As this technique is new and advanced so can also be used for various other future applications too.

Keywords- Covid 19, pandemic, SARS-COV-2, diagnosis, RNA Guided Endonuclease (RGEN), RT PCR, CRISPER-Cas-9, antiviral therapy

I. INTRODUCTION

The word "corona" is derived from a Latin word *corona* that means "crown". Hence corona viruses can be called as the viruses having crown like spikes on its surface. The first case of this virus was reported in Wuhan, China on December 8, 2019. This virus is responsible for causing the disease Covid-19 (named by WHO). This virus also shows resemblance with acute respiratory syndrome coronavirus (SARS-COV) therefore termed as SARS-CoV-2 by ICTV (International Committee on Taxonomy of Viruses). Within a very limited time duration Covid-19 was declared to be a Public Health Emergency of International Concern (PHEIC) on January 30, 2020 and pandemic on March 11, 2020 by WHO. The two genetic features of SARS-CoV-19 which has been reported was [1] Mutation in receptor binding domain of spike protein. [2] Introduction of polybasic cleavage site at the junction of two subunits of spike protein i.e., S1 and S2. SARS-CoV-2 is basically a single stranded RNA (ssRNA) virus and is highly contagious to human beings. It belongs to the genus *Betacoronavirus* and family *Coronaviridae*. The genome of SARS-CoV-2 is approximately 29.9 kilobasepairs in length and codes for 4 structural proteins i.e., spike protein (S), envelope protein (E), membrane protein (M) and nucleoprotein (N). The genome of this virus encodes a polybasic cleavage site in the spike protein which contributes to viral pathogenicity and transmissibility. The S, E and M proteins form the surface protein which are present on the viral envelope and responsible in causing infection in the susceptible host cells. The transmission of this contagious virus is aerosol mediated which originates during an affected individual's coughing and sneezing. The incubation period of this virus ranges from 2-14 days. The symptoms of it are fever, sore throat, breath shortening. In certain cases, it has also been identified that some infected individuals from covid19 are asymptomatic. Sometimes the symptoms depend upon the strain of virus also as the virus is very fast at mutating itself and forming a new variant. Due to the presence of variety of symptoms, the accurate diagnosis of this virus has become a challenge to reduce the mortality rate of people suffering from it. Currently SARS-CoV-2 is detected using RT-PCR (validated by WHO) but as it's been proven that the virus's genetic material is RNA and its mutation and evolution rate is too high, it may render the present diagnostic tools inefficient in due course of time. Also, the vaccines that have developed till date by different countries are not 100% effective against the virus, hence the accurate diagnosis of this disease has become a very important aspect in treatment of patients suffering from covid-19. The devastating effect of SARS-CoV-2 across the world demands a better management strategy that include both diagnostic as well as therapeutic tools.

The use of CRISPER-Cas technology could be an effective and novel approach to target viral RNA for its degradation and thus limiting the replication of the virus in host cells, resulting in its transmission control. The CRISPER-Cas system has been identified as a part of adaptive immune system in archea and bacteria against the infection of virus. Its applications in humans were found later. This system is a very novel tool to

develop therapeutics for the elimination of viral infections. In variety of other aspects also the CRISPER-Cas technology has been used such as treatment of HIV using antiviral therapy (although it was not cured in first attempt but was safe to use), treatment of cancer, treatment of blindness. Before this, CRISPER Cas has also been used for the detection of other RNA viruses such as lymphocytic choriomeningitis virus, influenza A virus and vesicular stomatitis virus.

II. BIOLOGY OF CRISPER CAS9 SYSTEM

The CRISPER- Cas9 system is an RNA based g system which helps in cleaving the double stranded DNA and providing an efficient method to induce deletions, insertions and specific changes to defined target sites. The editing of genome in a wide variety of cells can be performed by using CRISPER Cas9. Modified versions of the technology have added extra functions to the gene editing tools such as regulation of endogenous gene expression, modulation of epigenetic status and the labelling of genomic loci. This system of CRISPER- Cas9 was adapted for use in 2012. It is also used to correct pathological mutations in human embryos. The CRISPER-Cas9 caught the attention in scientific studies and research due to its unique adaptive nature and therapeutic potential. It is also faster, cheaper, efficient and more accurate than other existing genome editing methods.

CRISPER is found in Approximately 50% of sequenced bacterial genomes and nearly 90% of sequenced archea. In prokaryotes the CRISPER-Cas9 system is found to be the one and only adaptive immune system known so far. It is a naturally occurring genome editing system found in bacteria. The bacteria basically capture small pieces of DNA from invading viruses and use them to create DNA segments called as CRISPWE arrays. This CRISPER array allows the bacteria to remember the viruses. Hence if the virus encounters again then bacteria produce RNA segments from the CRISPER arrays to target the virus's DNA. The bacteria then use Cas9 enzyme to cut the viral DNA apart which then disables the pathogenic activity of virus. This system includes small guide RNAs used for sequence specific interference with invading nucleic acids. CRISPER-Cas9 comprises a genomic locus called CRISPER array that contains short repetitive elements (often palindromic) called repeats (35-45 bases). These repeats are separated by unique sequences called spacers (30-40 bases) which can take its origin from certain mobile genetic elements such as bacteriophages, transposons (jumping genes) or plasmids. The CRISPER array is preceded by an AT- rich leader sequence and is flanked by a set of cas genes encoding the Cas proteins. Basically, Cas9 is an enzyme that uses CRISPER sequences as a guide to recognize and cleave specific DNA strands that are complementary to CRISPER sequence. Hence Cas9 enzyme along with the CRISPER sequences form the basis of CRISPER-Cas9 technology. The immune response provided by CRISPER Cas system includes basically 3 stages i.e., adaptation, pre-CRISPER RNA (crRNA) expression/processing and interference. The initiation of adaptation stage is with expression of a complex Cas proteins by CRISPER-Cas9 loci and binding of these Cas proteins to the target DNA sequence, followed by two double strand breaks in the target DNA based on the Photospacer Adjacent Motif (PAM) which is a distinct short motif of 2-4 bases. The released segment of target DNA (photospacer) is inserted between two repeats of the CRISPER array and then acts as a new spacer. The CRISPER array transcription occurs in expression processing stage to generate a single long pre-crRNA. This is being followed by interference stage in which the mature crRNA bound to the processing complex and acts as a guide RNA to identify similar sequences in the invading viral RNA which is then cleaved and inactivated by one of the Cas proteins. The CRISPER Cas system can be classified into different types and sub types depending upon the occurrence of effector Cas9 proteins that convey immunity by cleaving foreign nucleic acids.

There are two classes of CRISPER-Cas9 systems i.e., class I and class II. Each class is classified into different sub-classes i.e., type I, III and IV for class I and type II, V and VI for class II. In class I system the RNP (Ribonucleoprotein) complex contains multiple protein subunits along with crRNA, while in case of class II system it contains only one protein and crRNA. The Cas9 protein in type II system with the help of tracrRNA and RNase II processes the pre-crRNA. The Cas12 and Cas13 proteins of type V and VI systems respectively processes the pre-crRNA themselves. The cleavage of the double stranded target DNA on the basis of recognition by mature crRNA occurs by the help of Cas12a and Cas12b proteins. While the target ssRNA is cleaved by the Cas13 protein. As compared to Cas12 protein, the Cas13 protein does not require any adjacent PAM in the target dsDNA (but not in ssDNA). A comparison between the nuclease characteristics of Cas12 and Cas13 is given in Table 1. When the complementary crRNA binds to the target RNA then it activates the Cas13 protein which then leads to the degradation of collateral ssRNA. This special property of the Cas13 protein has been used for the diagnosis of RNA virus infections similarly the Cas12 proteins can be used for the detection of ssRNA viruses. The Cas13 family consists of two HEPN domains that confer RNase activity and exists in four subtypes i.e., Cas13a, Cas13b, Cas13c and Cas13d. Among all these subtypes the Cas13d is most important because of its efficient and robust knockdown efficiency and easy viral delivery due to the small coding sequence of its effector domain.

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Table 1

Nuclease Characteristics	Cas12a	Cas13
PAM Required	Yes	No
PAM Identity	TTTV in dsDNA	Not Applicable
Cleavage	Single, Staggered cut	Multiple Cleavage Sites
Target Type	ssDNA, dsDNA	ssRNA
Collateral Activity	Yes	Yes

For the use of CRISPER-Cas9 system in lab, we shall have to create a small piece of RNA with a short guide sequence that binds to a specific target sequence of DNA in a genome. The RNA also binds to the Cas9 enzyme. Once the DNA is cut, we can use the cell's own DNA repair machinery to add or delete pieces the pieces of genetic material or to make changes to the DNA by replacing an existing segment with a customized DNA segment. Still the research is going on to find out the treatment or cure of various human diseases such as cystic fibrosis, haemophilia, sickle cell anaemia, cancer, heart diseases, mental illness and HIV (Human Immunodeficiency Virus) by the help of CRISPER-Cas9 technology. If we take a look on other genome editing techniques then those are only limited to the somatic cells (cells other than egg and sperm cells). Changes or modifications done in somatic cells are not inherited from one generation to another hence genome editing done in somatic cells can't be considered as much efficient. But if we talk about genome editing in germline cells the there are several ethical challenges arises including whether it would be permissible to use this technology to enhance normal human traits such as height or intelligence. So based on concerns about ethics and safety, germline cell editing is currently considered as illegal in many countries.

Applications of CRISPER Cas in therapeutic modality against infections caused by ssRNA viruses:

Since the outbreak of the novel Corona virus, many pharmaceutical and biotechnology companies have begun their intensive researches on the different therapeutic strategies such as production of repurposed antiviral drugs, vaccines and monoclonal antibodies to prevent the spread of this disease as well as to treat the infected individuals. Among the various strategies, advanced therapeutic approaches including cell and gene editing bases therapeutics are also being investigated and the initial results of in-vitro studies has been coming out to be promising as well, but still further assessments are also required. Scientific community across the world have also adopted this technology to eliminate viruses in human cells. Recently at Broad Institute of the Massachusetts Institute of Technology and Harvard University, USA demonstrated the potential use of Cas13 enzyme in the cell culture to inhibit the replication of three ssRNA viruses i.e., lymphocytic choriomeningitis virus, influenza A virus and vesicular stomatitis virus. It had also been found that the crRNA directed Cas13 enzyme is responsible for the efficient reduction of viral RNA in mammalian cells. Also, the Cas13 could process the CRISPER array to yield individual crRNA for targeting multiple viral RNA. It had also been found that the Cas9 enzyme was responsible for the induction of mutations in the target sites by CRISPER bases antivirals while treating the same with Cas13 enzyme does not showed any crRNA target site mutation. Hence, from all these reports and experiments it has been concluded that the Cas13 enzyme has potential to act as an effective antiviral to manage the SARS-CoV-2 infection.

Applications of CRISPER-Cas as a therapeutic modality with regard to COVID-19 and future prospects:

Taking into consideration the clinical use of CRISPER-Cas9, the most of its use in clinical aspects has been focused on to be ex-vivo gene editing of cells followed by their re-introduction back into the patient. However, in-vivo use of CRISPER technologies can be found to be problematic such as off-target editing and delivery and stimulation of counterproductive immune responses. As of now we all know that any specific therapy related to the cure of Covid-19 is not available, also the vaccines can't be called as 100% effective in preventing the Covid-19. Recently, CRISPER-Cas13 has emerged a potent system which is having the ability to protect the host bacterial cells against bacteriophage infection by using sequence specific crRNA. Concerning to this, a similar strategy may also be applied to design a therapeutic agent to target the ssRNA genome of SARS-CoV2 for managing covid-19. Recently the use of CRISPER Cas13d system has been implemented to target RNA molecules and this system can also be used for degrading SARS-CoV-2 RNA genome by designing crRNAs targeted to the ORF1ab (replicase-transcriptase) and S (spike) genes of the virus. As the amino acid sequence and molecular structure of RdRp gene of SARS-CoV-2 virus is highly conserved hence it can be an optimal target for CRISPER-Cas13 mediated virus RNA degradation. The most distinct feature of Cas13d enzyme is that its cleavage activity does not depend on PAM like sequences and this is the feature of it which facilitates the targeting of constantly evolving virus variants by rapid development of crRNAs. The delivery of this Cas13d effector was supposed to be via recombinant Adeno Associated Virus (AAV) in the covid-19 affected patients. The small size of the Cas13d

effector is expected to facilitate the AAV to carry the CRISPER array containing more than two crRNAs ensuring efficient virus clearance and reducing the probability of the generation of resistant strains.

A crisper-Cas13 based strategy for Covid-19 therapy termed Prophylactic Antiviral CRISPER in human cells (PAC-MAN) has been proposed by Abbott et al. in 2020 to inhibit the SARS-CoV2. This PAC-MAN approach could be a potential antiviral strategy to manage the emerging viral strains and could be implemented rapidly during a pandemic situation. The viral vectors such as AAV, adenovirus and lentivirus may be chosen as vehicles for the delivery of CRISPER based antivirals. The CRISPER-Cas13 based systems targets highly conserved regions in the viral genome thus enabling the system to act against the rapidly mutating strains of the virus.

Applications of CRISPER-Cas Technology in the diagnosis of covid-19:

As compared to those of RT-PCR techniques, the CRISPER-Cas based diagnostic tools are considered as to be more sensitive and specific. The use of this CRISPER-Cas9 technology in the field of Molecular Biology and diagnostics has induced a major change in today's era and in all the healthcare systems. The CRISPER-Cas system has also been applied to develop CRISPER-Cas9 mediated lateral flow nucleic acid assay (CASLFA) which has been used for the detection of pathogens. After this pandemic of covid-19, the requirement for the quick and easy diagnostic tool for detection has become a very crucial thing. Hence here the CRISPER-Cas tool has become very significant as its disease detection efficiency is within 30-60 min. Recently developed CRISPER based tools using Cas12a or Cas13a nuclease have been following the principle of "collateral cleavage activity". Cas12a/Cas13 nuclease, gets activated after the CRISPER-RNA (crRNA) targeted cleave and once activated, it cleaves all the nearby ssDNA or ssRNA molecules non-specifically. This feature is also called as collateral cleavage or trans-cleavage. The scientists have used this property and created fluorescently labelled ssDNA and ssRNA reporter probes to detect visible bands through the lateral flow assay in a paper strip, making it possible to develop a novel nucleic-acid based diagnostic tool. The crRNA targeted to the viral RNA could activate the Cas protein, leading to the collateral cleavage of the reporter probes and subsequently the appearance of a positive band on the paper strip.

A technology called Specific High-sensitivity Enzymatic Reporter Unlocking (SHERLOCK) technology has been invented recently and it utilises the crRNA-Cas13a protein activity to accurately identify the RNA molecules and cleaves collateral RNA along with target RNAs. This technology basically uses the non-targeted reporter RNA tagged with a fluorescent dye for the identification of specific RNA molecules. This CRISPER-Cas13 based SHERLOCK system has been used to detect the SARS-CoV-2. This system basically searches the virus-specific nucleic acids and provides a visual readout using a test paper strip within an hour. This SHERLOCK technology involves the amplification of the pathogenic RNA through reverse transcriptase recombinase polymerase amplification (RT-RPA) followed by in-vitro transcription to generate the corresponding ssRNA. The recognition of ssRNA by the Cas13-crRNA complex helps in activating the Cas13 nuclease which further exhibits cleavage of fluorescence tagged reporter ssRNA which is then analysed by lateral flow assay on the paper strips. DNA endonuclease targeted CRISPER trans-reporter (DETECTR) is a technique that amplifies the pathogenic DNA through isothermal recombinase polymerase amplification (RPA) and similar to the SHERLOCK system. For covid-19 diagnosis two specific crRNAs targeted to the E and N genes has been chosen along with a detection range of 70-300 copies per microliter of sample input. Loop mediated isothermal amplification (LAMP) technique with reverse transcription is used for the isothermal amplification step instead of RPA based amplification technique. This reduced the diagnosis time of covid-19 to just 30 minutes. As all the steps involved in this diagnosis were very sophisticated and it needs a grater care and the chances of contamination are also high in performing this diagnosis hence the care must be taken while performing it. Due to this high risk of contamination an All-in-one dual CRISPER-Cas12a (AIOD-CRISPER) technique came into use which is highly specific and faster in detection of viral nucleic acids. In this the reagents that are required for the detection of viral nucleic acids are incubated at 37 degree celsius which simplifies the detection procedure and also reduces the chances of contamination. This assay does not require the lateral flow-based paper dipstick.

III. DELIVERY OF CRISPER TOOLS

The therapeutic applications of the CRISPER technology include. the delivery of various tools inside the target cells under the in vivo condition. For the delivery of these tools the most preferable vehicles are the viral vectors. Several researchers also choose the Adeno-associated Virus (AAV) for the delivery of tools but these AAV viruses show limitations in the size of foreign genes to be carried by them. As the Cas proteins have a high molecular weight hence it is difficult for AAV to carry the gene encoding the Cas proteins along with the crRNA. This challenge has been overcome by the development of the low molecular weight Cas proteins. Though the viral vectors are seeming to be very beneficial for the delivery of CRISPER tools but there are also certain disadvantages of using this tool because it has been observed that the in in-vivo delivery by using the viral vectors there arises many issues such as immunogenicity. So, taking this in consideration, there

are several non-viral vectors are also available. These non-viral vectors are basically based on the use of lipids and inorganic particles. Hence these can be utilised as an alternative for viral vector mediated delivery of CRISPER tools.

IV. CONCLUSION

The rapid development of gene editing technologies within the last decade is providing significant advances towards improving human health. Various methods of gene editing are currently in development process for treatment of various kinds of diseases such as HIV, cancer, blood disorders etc. As per the current prevailing situation of the covid-19, the requirement for the specific and rapid tools and techniques for the diagnosis of the disease has become a very big challenge to the whole world. Also, since this virus generates normal cold & cough and flu-like symptoms, and sometimes the affected person remains asymptomatic too hence the accurate diagnosis of it has become very necessary to prevent this disease from spreading further to large mass of population. If the affected individuals are not properly screened then it's a very difficult task to vanish this pandemic disease from the world. Hence to stop this chain of spread the availability of an affordable and portable diagnostic system is required. Talking about the CRISPER Cas9 technology of gene editing, it has been used to generate diverse modifications in mammalian cells, including targeted editing of specific DNA sequences, activation or repression of genes of interest and epigenetic reprogramming of cellular identities. CRISPER-Cas system is a next generation technology helping in to develop novel highly specific anti-viral therapeutics and molecular diagnosis with rapid, accurate, point-of-care use without any need for technical expertise and expensive equipment in resource poor setting. CRISPER based diagnosis could fulfil this requirement being easy to use, portable and less time than RT-PCR based diagnosis method, but in current scenario the diagnosis of covid-19 is performed by RT-PCR only (by detecting viral RNA particles from respiratory samples) because the CRISPER based diagnosis and therapies are still not approved. CRISPER-Cas technology has also proved to be highly effective in diagnosis of some other viruses like dengue, zika etc. Diagnostics based on CRISPER-Cas technology like DETECTR has been shown to be fast, cost-effective and reliable. Also, the development of the therapeutic drugs against the RNA viruses is a major challenge because in RNA viruses keep on mutating their genome in every favourable way, leading to the development of anti-viral resistance in them. To face this challenge, the strategies have been explored for the development of therapeutics targeting CRISPER-Cas technology which not only just targets the viral genome but also the host machinery involved in virus replication. The host cell can be can be made resistant to SARS-CoV2 by delivering the CRISPER-Cas13d into the susceptible host cells. These proposed strategies of CRISPER based technology for covid-19 diagnosis are next generation technologies with significant challenges in terms of safety, delivery and regulatory approval for use in human subjects. Delivery of CRISPER tools remains the biggest obstacle in the use of CRISPER based therapeutics. Several methods also been proposed for the delivery of CRISPER tools and among all the various methods available for targeting nucleic acids, delivery of ribonucleoproteins rather than plasmid DNA or mRNA is associated with minimal off-target effects. Still there are various assessments associated with the use of CRISPER-Cas technology are pending and will be important for future clinical advancements.

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