Review Article

CRISPR-cas Methods : Culminating in Crescendo of the COVID-19 Pandemic to FELUDA Test

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COVID-19 pandemic is a universal crisis at this very moment. Since 31st December, 2019 and as of 30th November 2020, 63,187,035 cases of COVID-19 have been reported including 1,467,284 deaths. While Nucleic acid amplification-based methods; particularly real-time RT-PCR remains the gold standard for the diagnosis of COVID-19, various other diagnostic strategies are on trial to find rapid as well as sensitive and feasible testing technique. One such technique using CRISPR-cas based gene editing method is aimed at developing cheap, easy-to-use and fast SARS-CoV-2 detection kit for early diagnosis of COVID-19. CRISPR-cas methods for precise genome editing took its shape in modern form through the works of Dr. Jennifer Doudna at the University of California-Berkeley and Dr. Emmanuelle Charpentier at Umeå University in Umeå, Sweden and used for gene therapy, gene regulation, medical diagnostics and therapeutics. For this contribution to revolutionized genome editing, both of them had the honour to share the Nobel Prize in chemistry this year.

This review article tries to capture this colourful history in a single frame starting from the sequencing of 1.7 kbp *E coli* DNA fragments in the late 1980s by Y Ishino and detection of the first CRISPR sequence of *E coli* in 1987, subsequently unveiling the mysteries of in veritable adaptive immune system of prokaryotes against bacteriophages and plasmids, to the development of cheap and easy-to-use SARS-CoV-2 detection kit for early diagnosis of COVID-19.

This article also reviews various applications right from food processing to drug processing, diagnosis of infectious and non-infectious diseases, therapies for genetic abnormalities and even cancer through novel pathways of gene editing and regulation. Use of it as an antimicrobial and antiviral agent has also been elaborated. Finally, it has been discussed how this novel technique has been utilised for designing FELUDA, a CRISPR-cas9 based detection of COVID-19.
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Dr Jennifer Anne Doudna at the University of California-Berkeley and Dr. Emmanuelle Marie Charpentier at Umeå University in Umeå, Sweden worked across the Atlantic Ocean to elucidate the mechanism behind the CRISPR-*cas9* system in bacterial immunity and innovate adapting this system for precise genome editing. For the seminal contributions, both of them had the honour to share the Nobel Prize in Chemistry this year. This year, 2020, is a nightmare to all of us for the devastating pandemic waves submersing our lives at stake. Using these CRISPR-*cas* methods, many scientists, at different research institutes, independently developed cheap and easy-to-use SARS-CoV-2 detection kit for early diagnosis of pre-symptomatic and early symptomatic cases of COVID-19 to minimise chances of transmission in the community and to initiate adequate treatment ab initio to mitigate further complications. Thus, this year signifies a great coincidence of recognition to the contributors, as well as relief to the mankind from the threat of the pandemic, only through these CRISPR-*cas* techniques.

In short, CRISPR-*cas* systems, evolved in bacteria as an inheritable adaptive immune mechanism to confer protection against invading bacteriophages and plasmids

Editor's Comment :

- Clustered Regularly Interspaced Short Palindromic Repeats(CRISPR) — short palindromic repeats in gene sequences — was accidentally found to be involved in an inheritable adaptive immune mechanism in bacteria to confer protection against invading bacteriophages and plasmids through the activities of two main molecule types— a *cas* nuclease to cleave dsDNA and a gRNA to target specific viral DNA sequences.
- Through extensive work across the globe CRISPR-cas systems have been recognised as a very effective novel pathways of gene editing and regulation. Since then, there have been myriads of application right from food processing to drug processing, diagnosis of infectious, including recent COVID-19 and non-infectious diseases, including genetic disorders, therapies for genetic abnormalities and even cancer. Many more newer applications are ushering day by day at every nook and corner of the scientific world.

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through the activities of two main molecule types— a *cas* nuclease to cleave dsDNA and a gRNA to target specific viral DNA sequences, have myriads of application right from food processing to drug processing, diagnosis of infectious and non-infectious diseases, therapies for genetic abnormalities and even cancer through novel pathways of gene editing and regulation.

Aims and Objectives

To review the origin and development of CRISPR-*cas* system.

To study emerging applications of CRISPR-*cas* in basic and applied research.

To understand the integration of these know-how into daily practice.

A Puzzling Sequence from Bacteria Challenges the Early Sequencing Methodology

In 1987, Y Ishino *et al* in Osaka University sequenced 1.7 kbp *E coli* DNA fragments spanning over the *iap* (isozyme of alkaline phosphatase) gene region, and found that the same sequence appearing several times in different clones, downstream of the translation termination codon of the *iap* gene by conventional M13 dideoxy sequencing and autoradiography. Similar sequence were detected in other *E. coli* strains (C600 and Ymel), members of *Enterobacteriaceae*, *S. dysenteriae*, *S. enterica typhimurium* by Southern blot hybridization analysis; and also in *Actinobacteria*, e.g. *M. tuberculosis.* About fifteen years later, such repetitive sequence has been termed CRISPR.

Discovery of CRISPR in Archaea

Francisco J. M. Mojica, Cesar Díez Villaseñor, Elena Soria of Universidad de Alicante and Guadalupe Juez of Universidad Miguel Hernández, in January 2000, demonstrated transcription of genomic regions having the repeated sequences in extremely halophilic archaea, *H. mediterranei* and suggested regulation of gene expression in conversion of B to Z form of DNA, which is barely valid for bacteria;

Observations of such repeated sequences interspersed with variable sequences in various bacterial (H. Influenza, S Cerevisiae,-*Methanocaldococcusjannaschii*) and archaeal genomes (*H. mediterranei*, *H. Volcanii)* by many scientists across the globe led them to name such sequences differently as Short Regularly Spaced Repeats (SRSR), Spacers Interspersed Direct Repeats (SPIDRs), and Large Cluster of Tandem Repeats (LCTRs) – two groups of LCTR sequences were observed in hyperthermophilic archaea, *P. abyssi* and *P. horikoshii*, with presumed role in chromosome partitioning; but scrambling of numerous such repeats in the genome of *P. furiosus* frowned at this assumption. [Zivanovic *et al*., 2002]

Mojica *et al* (2000) pioneered with the concept of functional relationship among these repeated sequences in the genome of various bacteria and archaea and coined the term CRISPR, through correspondence with Ruud Jansen; but Jansen et al. first used the term Clustered Regularly Interspaced Short Palindromic Repeats(CRISPR) in print version in 2002.

Comparative genomic studies focussed at the common characteristics of CRISPR as their intergenic locations, multiple short direct repeat sequences with meagre variation and nonconserved interspersed sequences and a common leader sequence of hundreds of bps at one side of the repeat clusters. CRISPR sequences have been demonstrated in archaeal genomes and 50% of bacterial genomes but none in eukaryotic genome till date.

Identification of *cas* Genes

At the onset of new millennium, four conserved genes were found to be present regularly juxtaposed to the CRISPR regions and were termed as CRISPR-associated genes (*cas 1, 2, 3 and 4*). *cas 3* was having the seven motifs peculiar to the superfamily 2 helicases and *cas 4* was found to be working as a part of RecBCD complex for the terminal resection of the double strand breaks to initiate homologous recombination, related to RecB exonucleases. Thus *cas 3* and *cas 4* were thought to be operational in DNA repair and recombination, transcriptional regulation and chromosome segregation. But, *cas 1* and *cas 2* were not identified with similarity to functional domains of any known proteins. [Jansen *et al*, 2002] Simultaneously, Makarova *et al.* (2006) independently and systematically analysed the conserved genes in all available prokaryotic genome and found multiple clusters of genes corresponding to *cas* genes (encoding putative DNA polymerase, helicase, RecB-like nuclease) in genomes of hyperthermophilic archaea and in two hyperthermophilic bacteria, *Aquifexaeolicus* and *Thermotoga maritima* but not in mesophilic and moderate thermophilic archaea and bacteria, predicting these proteins to be a part of some 'mysterious' ill-defined DNA repair mechanism in thermophilic organisms.

Discovery of CRISPR Function

Independent breakthrough observations—by Francisko Mojica in Alicante and Christine Pourcel in Orsay—of interspersed or spacer regions between repeat sequences homologous to sequences of bacteriophages, prophages and plasmids and background knowledge of immunity against phages and plasmids by the host strains harbouring the homologous spacer sequences in the CRISPR led to the proposition of CRISPR-mediated biological defence system similar to eukaryotic RNAi system from the entry of foreign mobile genetic elements. Both Mojica *et al* and Pourcel *et al,* in 2005, independently

suggested some triggering mechanism by CRISPRs to form a memory of previous genetic aggressions. In 2005 itself, Bolotin *et al* confirmed these observations by further finding of a correlation between number of spacer of phage origin and degree of resistance to phage infection and suggested possible production of anti-sense RNA using CRISPR.

Seminal works over decades by... Makarova et al. (2006), Barrangou *et al* (2007), Marraffini et al. (2008), Brouns et al. of van der Oost's group (2008), Moineau et al. (2010), Siksnys' group (2011), Andersson and Banfield (2018) have elucidated many intricate details of CRISPR-*cas* systems and reinforced our good understanding of their precise details[1-4].

Cas1 and *cas2* are the conserved protein in most of the CRISPR-*cas* system, representing the adaptation module for insertion of new spacer in the CRISPR arrays. During expression stage mature crRNAs are processed by type-specific *cas* endonucleases from pre-crRNA which are transcribed from CRISPR locus. The crRNAs are bound by effector *cas* endonucleases and recruited to and sequentially cleave the target DNA or RNA, during the interference stage. In contrary to the adaptation module, *cas* enzyme are variable from one CRISPR-*cas* type to another, while involved in the above two stages but with participation of the same enzymes.

Diversity and Classification of CRISPR-*cas*

CRISPR contents and distribution varies strikingly even in closely related strains, eg, *M tuberculosis* has CRISPR but not *M. leprae*. Contrarily, *E coli* and *M avium*, as well as *Methanothermobacto-rthermautotrophicus* and *Archaeoglobusfulgidus*, although being phylogenetically distant, contain nearly identical CRISPR sequences. There are 1 to 18 CRISPR arrays in one genome and 2 to 374 repeat units in one CRISPR array.

According to the latest (09 May, 2017) CRISPR database[5], CRISPR were identified in 87% (202) of 232 analysed archaeal species and 45% (3059) of 6782 analysed bacterial species. But peculiarly, CRISPR-*cas* system as found to be much less (around 10%) prevalent in wild microbial communities in a survey of 1724 draft genomes, might be due to lack of CRISPR-*cas* systems across majority of uncultured bacterial lineages[6].

Shmakov *et al*, in 2017, classified CRISPR-*cas* system in two distinct classes: Class 1 (nearly 90% of all identified loci) — included types I, III and IV, widespread in bacteria and archaea, including all hyperthermophiles and working with multisubunit effector complexes having unevenly stoichiometric 4 to 7 *cas* proteins and Class 2 (remaining 10%) — included types II, V and VI, almost exclusively spread in bacteria and working with a single multi-domain effector protein.

Unit signature proteins, like *cas3*, *cas9* and *cas10* distinguish type I, type II and type III respectively. Architecturally similar and evolutionarily related CRISPR-associated complex for antiviral defence (Cascade) and Csm/ Cmr complexes are multimeric effector complexes of type I and type III systems respectively[7-9]. Functionally, nonspecified type IV systems contain no adaptation module with *cas1* and *cas2* nucleases[10]. Effector modules of subtype III-B systems utilizing spacers made by type I systems stood for the modularity of CRISPR-*cas* systems. Albeit being devoid of identifiable CRISPR loci in many of the genome encoding type IV systems, it— alike subtype III-B— utilizes available crRNAs from different CRISPR arrays[10]. Lastly, additional signature genes and characteristic gene arrangements classify each type into multiple subtypes: I-A, B, C, D, E, F, U; III-A, B, C, D(of Class 1); II-A, B, C; V-A, B, C, D, E, U; IV- A, B, C (of Class 2)[8,11].

Class 2 Systems are Suitable for Genome Editing Technology

A new generation of genome editing technology attractively utilised simple architecture of effector complexes of Class 2 CRISPR-*cas* systems, which contain multiple distinct effectors like *cas9* in type II, *cas12a*(Cpf1) and *cas12b*(C2c1) in type V, *cas13a*(C2c2) and *cas13b*(C2c3) in type IV[8,11]. *cas9*, a crRNA-dependent endonuclease with two distinct nuclease domains, RuvC and HNH—respectively responsible for cleavage of nontarget (displaced/ non-complementary) and target (complementary) DNA strands in the crRNA target DNA complex—was the most commonly and best studied multidomain effector protein

Cas12a (Cpf1) present in several bacterial and archaeal genomes is a prototype of type V effectors containing two RuvC like nuclease domains but not HNH domain; although a second nuclease domain with a unique fold being functionally analogous to HNH domain of *cas9* has been reported recently[11,12]. *cas12a*, as a single RNA-guided nuclease, unlike *cas9* activity, is independent of a tracrRNA (*trans*-activating crRNA); and has different cleavage pattern and protospacer adjacent motif (PAM) recognition[13]. Like *cas9*, type V effectors also require a tracrRNA for the targeted activity. The group of Emmanuelle Charpentier, in 2011, besides finding *cas9* containing CRISP-*cas* system while sequencing small RNA of *S. pyogenes*, also discovered a second small RNA as tracrRNA, which guided *cas9* to its target by forming a duplex with crRNA. Unlike most of the functionally specified CRISPR-*cas* system reposted to target DNA, only multi-component type III-A and III-B systems also target RNA[14]. On the contrary, type IV effectors, *cas3a* and *cas3b*, containing a pair of higher eukaryote and

prokaryote nucleotide-binding (HEPN) domains instead of RuvC-like nuclease domains, specifically target RNA to mediate RNA interference[15]. Novel Class 2 effectors presumably paved CRISPR systems through a newer avenue to genome engineering technology[16].

Applications of CRISPR-*cas*

1. CRISPR typing: CRISPRs were initially used for diagnostic and epidemiological typing of *M. tuberculosis* and subsequently *Yersinia pestis, Salmonella sp., Corynebacterium diptheriae* using the property of heterogeneity of CRISPRs among isogenic isolates. An initial PCR-based method was improvised into spoliogotyping, a hybridization-based method, suitable for routine use and High Throughput (HTP) genotyping.

2. In dairy industry: Danisco (DuPont) first demonstrated immune function of CRISPR-*cas* and utilized that to yield phage resistant *S. thermophilus* in improved cheese production. [Barrangou *et al*., 2007]

3. Genetic tools for eukaryote cells: Development of the type II system, particularly by combining crRNA and tracrRNA into a sgRNA (single guide RNA) [Jinek *et al*, 2012] and deploying *cas9* system—unlike target-degrading *cas3*—produces a single DSB in the DNA as an important gene editing tool for genetic alteration in either of the two ways, (a) NHEJ and (b) HDR.

a. Non-Homologous End Joining (NHEJ), which joins the cut end but may cripple the gene product by deleting a few bases or may inactivate it by frameshift mutation.

b. Homology Directed Repair (HDR), which uses another piece of DNA with homology to the target to repair the damaged allele by inserting that DNA element either through recombination, insertion, deletion or changing sequence. [Cong *et al*, 2013; Mali *et al*, 2013]

Instead of previous complicated approaches using ZFNs (Zinc Finger Nucleases) and TALENs (Transcription Activator-like Effector Nucleases), [Mali *et al*, 2013] *cas9* is very simple to retarget and concurrently to modify several targets. Albeit the necessity of a PAM juxtaposed to the target *cas9* within a short spell of time has become a very popular tool for genome editing in studying eukaryotes, from yeast to humans. [Terns *et al*., 2014] *cas9*-based methods have also been applied in genetic screening, [Wang *et al*., 2014] and programmable RNA recognition and cleavage. [O'Connell *et al*., 2014] Strategies for prediction and prevention [Tsai *et al*., 2015] of off-target effects (interactions with unintended targets) by *cas9* are being developed, using an artificial CRISPR-*cas* nuclease RFN (RNA-guided FokI nuclease)[17].

4. Diagnostic uses:

a. CRISPR-based diagnosis of viruses—Methods based on the CRISPR-*cas12a* (DETECTR) and CRISPR-*cas13a* (SHERLOCK) families are the most widely explored arena for CRISPR-based diagnostic system.

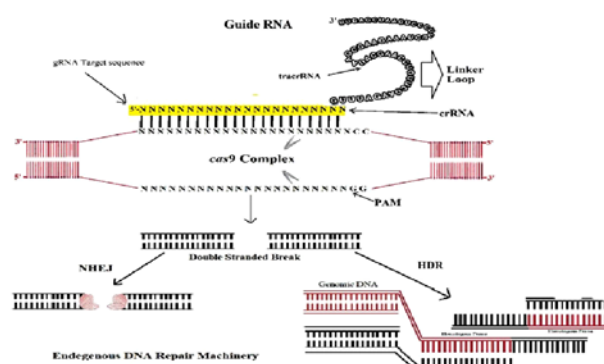

Fig 1: Schematic representation of gene editing by CRISPR-Cas9 complex.

The guide RNA recruits the complex at the desired site on the genome; the Cas9 protein cuts at the PAM site and then one of the two DNA double strand break repair mechanisms repair the break leading to gene editing.
Non-homologous end joining (NHEJ) is a pathway that repairs double-strand breaks in DNA, where the break ends are directly ligated without the need for a homologous template, in contrast to homology directed repair, which requires a homologous sequence to guide repair.
Homology directed repair (HDR), a naturally occurring nucleic acid repair system, can be used to modify genomes in many organisms, including humans, which is initiated by the presence of double strand breaks (DSBs) in DNA.

i. DETECTR—A guide RNA primarily leads the type V *cas12a* enzyme to a ds sequence of DNA in a specified viral genome; then *cas12a* enzyme indiscriminately cleaves a quencher-molecule-bound ss DNA molecule and a reporter fluorophore, emitting a fluorescent signal from the fluorophore and quencher. This highly sensitive DETECTR method can detect a single molecule of viral particle, within a µl of sample[18,19]. DETECTR may detect any viral particle but most significantly used for diagnosis of HPV (Human Papilloma Virus)[20].

ii. SHERLOCK—Using crRNA targets via type IV *cas13a* enzyme, a target RNA-bound targeting molecule along with an attached fluorophore are cleaved indiscriminately and emit fluorescent signal in presence of specific sequences of viral nucleic acid through simplified and more specific SHERLOCKv2 protocol. The sensitivity can be enhanced over three times by supplementing *cas13* enzyme with CRISPR-associated *Csm6* enzyme. SHERLOCK protocol can be optimized for diagnosis of HIV (Human Immunodeficiency Virus). To enhance amplification and detection of viral material, recombinase polymerase amplification (RPA) may be united with either of SHERLOCK and DETECTR[19-22].

Heating unextracted diagnostic samples to obliterate nucleases (HUDSON) protocol escalates the efficiency of SHERLOCK procedure to detect genetic material from urine, saliva, blood and its isolates. Conserved region within the genetic material of these viruses can be recognized using universal-flavivirus RPA and viral species-specific crRNAs. SHERLOCK technique with HUDSON protocols can detect any virus but flaviviruses like Zika, Dengue, West Nile and Yellow fever viruses have mostly been detected[21,22].

Presence of SARS-CoV-2 in the specimen can be detected by identifying both of the N and E gene variants of the virus by DETECTR method[23], and both of the S and Orflab gene sequences by SHERLOCK method[24].

b. CRISPR-based diagnosis of bacteria—FLASHit is a software tool to design a *cas9* enzyme set to target a total of 3624 bacterial genetic sequences associated with microbial resistance including drug-resistant *S aureus* culture isolates. MRSA (Multidrug-Resistant *S Aureus*) and vancomycin-resistant *E faecium*. FLASHit is based on FLASH (Finding Low Abundance Sequences by Hybridization), using *cas9* enzyme recombination along with multiplex guide RNAs for precise identification of pathogen by eliminating background sequences and the *cas9* system to cleave target sequences into fragments ideal for next generation sequencing. [Quan *et al*, 2019]

FLASH method can rapidly detect Mtb using RPA and *cas12a*-optimized enzyme. [Ai *et al*, 2019] With a 99.06% sensitivity rate, FLASH method can detect hybrid strain of STEC O104:H4.

c. CRISPR-based diagnosis of non-infectious diseases—CRISPR method effectively sought for specific oncogenes related to almost all cancer cells in the body and could successfully identify synergistic gene reaction behind drug-resistance for better understanding molecular pathophysiology of cancer and identifying new precision therapy biomarkers. SHERLOCK method using *cas13* enzymes thus helps in early and precise diagnosis vis-à-vis prevention of many cancer. [Khambhati *et al*, 2019; Tian *et al*, 2019]

5. Commercially available CRISPR-based diagnostic tools: Three CRISPR-based diagnostic systems or instruments are available commercially

a. DETECTR Diagnostic Tests—Manufactured by Mammoth Biosciences, using CRISPR type V and programmable for a wide array of viral, bacterial, infections and also cancer diagnosis. Like SHERLOCK method, DETECTR method has been proposed for detecting SARS-CoV-2.

b. SHERLOCK Diagnostic Tests—Manufactured by SHERLOCK, using CRISPR type IV and presented a cheap and faster method using paper strip, lateral flow, read-out assays for Zika and Dengue virus. Boosted with HUDSON protocol, the SHERLOCK method bypasses purification and dilution steps for rapid diagnosis of viral infections and in field studies.

c. CRISPR-*cas9* Products for Gene Editing—Manufactured by Sigma-Aldrich, using type II CRISPR-*cas9* systems for gene editing and diagnosis.

6. Therapeutic uses: CRISPR-*cas* systems have immense potential at therapeutic level, like

a. As an antimicrobial agent—Specifically to target antibiotic-resistant and/or highly virulent strains of bacteria. [Bikard *et al*, 2014; Citorik *et al*, 2014]

b. As Gene therapy—

i. By repairing the *cftr* gene in cultured cells from cystic fibrosis patients. [Schwank *et al*, 2013]

ii. By altering DNA in mouse germ cell lines to cure dominant cataract disorder and Duchenne muscular dystrophy. [Wu *et al*, 2013; Long *et al*, 2014]

iii. By genetic alteration in adult mice to cure hereditary tyrosinemia. [Yin *et al*, 2014]

A short version of *cas9* delivered through adeno-associated virus facilitate its use in somatic gene therapy. [Ran *et al*, 2015]

c. As an antiviral agent—Potential for treatment of viral infections, like HIV [Hu *et al*, 2014; Ye *et al*, 2014] and Hepatitis B [Zhen *et al*, 2015]

7. In disease modelling: Precise genetic modification through Gene editing in embryos of primates, close to human, allows development of disease model. This approach might be used to change DNA in human embryos to stop noncomplex hereditary diseases but not for complex trait, due to strong ethical issues. [Baltimore *et al*., 2015] *cas9*-mediated genome editing also accelerated the generation of transgenic models to expand biological research beyond traditional genetically tractable animal model organisms. This could be useful to develop novel transgenic models, [Wang *et al*., 2013] to engineer isogenic ES and iPS cell disease models with specific mutations introduced or corrected, respectively, or in vivo and ex vivo gene correction.[Schwank *et al*., 2013; Wu *et al*., 2013]

8. Gene regulation: Programmable gene regulation utilising CRISPR-*cas* systems have been developed for

a. Gene silencing by interfering with RNA-polymerase binding or elongation by using both Cascade and a nuclease-deficient *cas9* mutant (d*cas9*)[25,26].

b. Achieving transcriptional activation or repression by fusing d*cas9* with a transcriptional activation domain or repressor. [Cheng *et al*, Farzadfard *et al.* and Gilbert *et al*., 2013]

c. Achieving strong induction by adding multiple activity domains[27].

9. Genome-wide application of this system helps in identifying the genes behind treatment resistance of melanoma cancer cells.

10. Functional genomic screening: Unbiased genome-wide functional screen, through genome editing with *cas9* parrallely over many targets helps to identify genes behind a particular phenotype, eg, robust negative and positive selection screens in human cells by presenting loss-of-function mutations into early constitutive coding exons of a different gene in each cell. *Cas9*-mediated pooled sgRNA screens are more sensitive and consistent than older RNAi approach to

target almost any DNA sequence significantly obviating off-target effects and limitation of partial knockdown.[Wang *et al*. and Shalem *et al.* 2014]

11. Transcription modulation: CRISPRi (CRISPR-based interference) performs much better in prokaryotic genomes than eukaryotic cells and this repressive function of CRISPRi is further enhanced by linking d*cas9* to transcriptional repressor domains, eg, KRAB or SID effectors, to promote epigenetic silencing; even adding helper functional domain yields only partial transcriptional knockdown. Being fused with VP16/VP64 or p65 activation domains, *cas9* turns into a synthetic transcriptional activator[27].

12. Epigenetic Control: Instead of having achievement in locus-specific targeting of epigenetic modifying enzymes in a small number of proof-of-concept studies with the use of zinc finger proteins and TAL effectors as before, *cas9* epigenetic effectors (epi*cas9*s) can artificially add or remove specific epigenetic marks at specific loci more flexibly to find causal effects of epigenetic modifications in shaping the regulatory networks of the genome. Possible off-target effects or cross-talk between effector domains and endogenous epigenetic complexes should be cautiously specified and mitigated by harnessing prokaryotic epigenetic enzymes[27].

13. Live imaging of cellular genome: Alternative to DNA-FISH, a powerful live imaging technique using fluorescence-tagged cas9 labelling of specific DNA loci was developed recently. [Chen *et al*, 2013]

14. Inducible regulation of *cas9* activity: Bilobed structure of *cas9* can be split into two units and be reassembled under control via small molecule induction facilitating systemic control in patients or animal models or via light inducible heterodimeric domains, eg, CIB1 or CRY2 or chemically inducible analogues, eg, ABI and PYL, to construct inducible TALEs (Transcription Activator-like Effectors)[27].

Detecting SARS-CoV-2 by CRISPR-*cas* Method[28]

As have already described the detection of SARS-CoV-2 by CRISPR-*cas*-based SHERLOCK and DETECTR methods, the latter is more commonly used in RT-LAMP using *cas12*. COVID-19, due to SARS-CoV-2, is a devastating pandemic, jeopardizing economic foothold of many nations, social existence of the population thereof, financial independence of most of the citizens across the globe, is a real menace to the fruitful existence of mankind. Many pre-symptomatic carriers are the real threat to others for its transmission. Fast and cheap tool to detect the presence of the specific virus in any susceptible person, at anytime and anywhere, is the need of the hour.

DETECTR RT-LAMP/*cas12* (improvised) is like 'the magic lamp' in the present scenario. It is claimed to be equally effective as the gold standard diagnostic tool for SARS-CoV-2, i.e.qRT-PCR. Although qRT-PCR is a quantitative assay, it is a complicated procedure at altered temperature over a prolonged time with less temporal turnover. Whereas, qualitative assessment at normal temperature in a simpler way makes RT-LAMP a handy tool even for the novice, particularly when it is supplemented with microfluidic- or SPR (surface plusmon resonance) -based detection system, it becomes a transportable rapid test that can be applied at the patient's site.

Charles Chiu of the University of California-San Francisco, in collaboration with San Francisco-based biotech Mammoth Biosciences tried to develop a CRISPR-based diagnostic test for SARS-CoV-2 detection with their previous experience of developing similar test for Lyme disease. Chiu et al. developed SARS-CoV-2 DETECTR, for SARS-CoV-2 DNA endonuclease-targeted CRISPR trans reporter and published its mode of action in *Nature Biotechnology* on April 16, 2020. After RNA extraction, it uses Loop Mediated Amplification (LAMP) at isothermic condition avoiding expensive thermocyclers for cycling temperatures as in PCR study. On July 9, 2020, Emergency Use Authorization (EUA) was allowed by USFDA only for the use at the UC-SF's clinical lab. On May 20, 2020, Mammoth Biosciences allied with Glaxo SmithKline Consumer Healthcare to develop DETECTR into a handheld disposable instrument at a very cheap rate.

On August 27, 2020, *PLOS Pathogens* published a paper describing a test by a group of China-based researchers, named 'CRISPR-COVID', for RNA extraction, followed by cleavage of single stranded reporter using *cas13a* and having florescence in presence of SARS-CoV-2 RNA.

On August 31, 2020, researchers published in PNAS about a LAMP-based SARS-CoV-2 detection tool utilizing microfluidic cartridge and smartphone-based reader, avoiding laboratory-grade infrastructure and resources for diagnosis at the collection-point, like schools, sports arenas, old age homes, etc. On the other hand, Max Wilson of University of California-Santa Barbara developed a COVID-19 test that uses CRISPR for detection and PCR for amplification but avoided LAMP considering it to be less sensitive.

On May 6, 2020, COVID-19 test kit based on CRISPR using SHERLOCK platform by Dhanda et al. at Sherlock Biosciences was granted EUA and started distribution in the USA in collaboration with Integrated DNA Technologies. Wyss Institute at Harvard University developed INSPECTR (Internal Splint-pairing Expression Cassette Translation Reaction) through hybridization of a sample like saliva to cryosynthetic DNA complementary to SARS-CoV-2 RNA. In presence of viral RNA, a reporter protein is activated and can be

observed without any instrument, making it perfect for domiciliary use.

FELUDA : The Indigenous SARS-CoV-2 detection Kit[29]

On October 11, 2020, Dr. Harsh Vardhan, the then Union Health Minister of India, stated with high optimism that in the following couple of weeks the FELUDA paper strip test for SARS-CoV-2 detection would be available. He also acclaimed 96% sensitivity and 98% specificity of the test, based on trials at the Council of Scientific & Industrial Research-Institute of Genomics and Integrative Biology (CSIR-IGIB).

FELUDA (FNcas9 Editor-Limited Uniform Detection Assay), also after the name of a popular Bengali fictional private investigator character in the thrillers by Satyajit Ray, utilises CRISPR-cas technology for the detection of genes specific to SARS-CoV-2 virus with the help of a protein called FNcas9 and a guide RNA (gRNA). Using a paper strip, binding of this gRNA-FNcas9 complex to the SARS-CoV-2 viral gene could be visualized by simple coloured line(s) within a spell of one to two minutes. Single line indicates negative and double lines indicate positive test. It got approval from the Drugs Controller General of India (DCGI).

FELUDA test is very less expensive, very less time consuming, very simple to operate and interpret by the lay person as claimed by Dr.Debojyoti Chakraborty, Senior Scientist at CSIR-IGIB. He is one of the authors of the pre-print shared on bioRxiv that describes the development of FELUDA.

In May, 2020, CSIR IGIB and TATA Sons signed a MoU for licensing the know how related to development of the kit.

Discussion

Accidental finding of short repeats in genome sequences in a palindromic pattern drew the curious attention of young researchers in late 1980s. Sincere attempt to isolate the gene for a single protein (*iap* enzyme) led the scientists to dive deep into the gubernaculums. A mysterious finding of inheritable adaptive immunity with memory, similar to vertebrates, overwhelmed the scholars. Probing deep into it, they discovered a very peculiar mechanism of prokaryotic adaptive immunity against bacteriophages and plasmids, utilising those short palindromic repeats in gene sequences, namely CRISPR. This is the beginning of a new journey through a novel path towards gene editing technology, gene regulation, gene therapy, so on and so forth.

Prokaryotic adaptive immunity, as well as gene editing technique with minimum off-target effects, are through the CRISPR-*cas9* systems, whereas *cas12a*, *cas13a* systems help in detection of several viral and bacterial infections. Cheap and fast detection of SARS-CoV-2 using DETECTR or SHERLOCK platform of CRISPR techniques, including the indigenous FELUDA Test Kit, is a boon to the threatened world population during this dreaded pandemic. Fermenting milk to produce cheese with the help of CRISPR-mediated phage-resistant lactose fermenting bacteria is a bonanza to the food processing industry. Getting into the genetic link behind drug resistance in antimicrobials and getting over of it are the contributions of this novel technology. Finding specific oncogene, vis-à-vis cancer, biomarker and suggesting gene therapy for cancer and also understanding the molecular pathophysiology of different monogenic and polygenic diseases through cell modelling and animal modelling, even at the level of embryo with the help of CRISPR technology are of immense help for the medical fraternity and mankind. The CRISPR revolutionized genome editing, identifying right enzymatic system in a very simpler way and this is as significant as the discovery of thermostable DNA polymerase in PCR evolution.

The great inquisitiveness of human mind brings forth the latest generation of genome engineering tools from the component of microbial anti-phage defence systems. Similarly and very likely, the unabated human wisdom will get to the bottom of the amusing biological variety of nature to refine genetic modification in a more competent and precise way.

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