REVIEW ARTICLE

CRISPR Technology in Disease Diagnosis

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Abstract

CRISPR refers to Clustered regularly interspaced short palindromic repeats. These are palindromic sequences interspaced by viral nucleotide base pairs (spacers), which act as an adaptive immune gadget along with Cas proteins. Class 2 CRISPR-Cas systems are widely used for various applications due to the single Cas protein effector complex. It is widely used as a genome editing tool. In diagnostics, gRNA in conjunction with Cas proteins cut a specific sequence with complementarity and fluorescence is produced by the reporter molecule. This can be extensively used as a point-of-care diagnosis because of various advantages over other molecular techniques. In India, CRISPR utility is in the budding stage.

Keywords: Cas, CRISPR, Diagnosis, Genome editing, gRNA.

Ind J Vet Sci and Biotech (2023): 10.48165/ijvsbt.19.4.02

Introduction

CRISPR array consists of spacers and repeats, these are arranged in the manner of spacer-repeat-spacer-repeat and so on. The spacer contains the sequence of the foreign (bacteriophages, mobile genetic elements) DNA, while repeats contain palindromic sequences of bacterial host genome, so in CRISPR array repeats are interspaced regularly by spacers.

Cas proteins are CRISPR associated proteins. These protein sequences are always found near to the CRISPR array (Ishino *et al.,* 2018). Different Cas proteins are defined to perform a particular activity as part of a bacterial defense system. The Cas1 and Cas2 are associated with adaptation, similarly various other Cas proteins are involved with expression and interference activity. The major function of Cas proteins is its cleavage activity in the interference due to its nuclease activity, which cleaves the DNA strands when guided by gRNA (Makarova *et al*., 2020). This article explains applications of the CRISPR-Cas system in various fields with special reference to diagnosis.

Discovery

In the year 1987, a team of Japanese molecular biologists led by Y. Ishino was working on the sequence analysis of alkaline phosphatase isozyme in *Escherichia coli* (Ishino *et al.,* 2018). Upon sequencing they found the DNA repetitive sequences downstream of this gene. The repeat sequences were interspaced with unique sequences (Han and She, 2017). Later similar sequences were observed in other strains of *Escherichia coli* and other bacteria such as *Salmonella dysenteriae* and *Salmonella enterica* (Nakata *et al.,* 1989). A similar pattern was observed in *Mycobacterium tuberculosis* IS987 flanking sequences with direct repeats of 36 bp, interspaced by non-repetitive spacer DNA of 35 to 41 bp (Hermans *et al*., 1991). So this suggested that these sequences are found with closely related bacteria. Next, a team was working on the regulatory mechanisms of haloarchaea (*Haloferax mediterranei* R-4 strain) to survive salinity changes in seawater. At the time of analysis of sequences, they found an unexpected pattern of 30 bp DNA fragment repetitive at regular distances (Mojica and Rodriguez-Valera, 2016).

Subsequently, this CRISPR array was discovered in 40 species of prokaryotes. Another interesting finding observed was that the leader sequences were found on one side of the CRISPR loci which were within a few hundred base pairs from CRISPR loci. These sequences had 80% homology irrespective of the species of bacteria, and these genes were named CRISPR associated genes (Cas1 to Cas4) (Jansen *et al*., 2002).

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How to cite this article: Bhure, M., Chavan, K., Kalke, M.G., Shinde, S., Khan, W., Patil, A., & Chaudhari, S. (2023). CRISPR Technology in Disease Diagnosis. Ind J Vet Sci and Biotech. 19(4), 7-13.

Source of support: Nil

Conflict of interest: None

Submitted: 20/05/2023 **Accepted:** 10/06/2023 **Published:** 10/07/2023

Breakthrough in understanding the function of CRISPR was brought to the light by Mojica Francisco J.M. and Pourcel C. The two scientists individually found that spacer sequences had homology with sequences of bacteriophages. So they proposed these spacer sequences are memory of past infection and help to combat the invading bacteriophages (Mojica *et al*.. 2005; Pourcel *et al*., 2005). Later these observations were confirmed by Bolotin and colleagues by correlating between spacers of phage origin and the degree of resistance to phage infection (Ishino *et al*., 2018).

Classification of CRISPR-Cas System

The CRISPR-Cas system have been broadly into two categories comprising three types of each ,class 1 includes types I, III and IV; class 2 includes types II,V and VI. Class 1 system uses multisubunit complexes that contain multiple different Cas protein , whereas class 2 effector contains only a single protein. The variation in the different CRISPR-Cas system is due to exposure of bacteria to mutating viruses and anti-CRISPR strategies that make bacteria to evolve with these conditions (Takeuchi *et al*., 2012).

Cas proteins can be observed from 1 to 13 types. Cas 1, 2 and 4 are mainly associated with adaptation. While Cas 3, 5, 7, 10 are involved in interference activity and Cas 6 has expression activity in Class 1 CRISPR-Cas system. Cas 9, 12 and 13 has interference activity in Class 2 CRISPR-Cas system (Ishino *et al*., 2018).

Mechanism of CRISPR-Cas System

The mechanism in which CRISPR-Cas system works is explained with 3 stages, *i.e.,* adaptation, expression and interference (Fig. 1). Here the understanding of the different Cas proteins plays a vital role in understanding the mechanism.

Adaptation: Whenever any virus enters the bacteria for the first time, bacteria take up a part of viral genome and adopts into CRISPR array as a spacer sequence. Different Cas proteins are involved in the adaptation, Cas1 protein has sequence independent with metal dependent endonuclease function for DNA, while Cas2 has metal-dependent ssRNA endonuclease activity and prefers for U-rich fragments (Al-Attar *et al*., 2011). These 2 along with Cas4 recognise the proto-spacer and cleave the unique viral sequence and integrate into the CRISPR array of host genome (Lee *et al.,* 2019).

Expression: When the virus enters next time, the CRISPR array undergoes transcription to form precursor crRNA (pre-crRNA), this pre-crRNA attains maturity and form crRNA for different spacers that are acquired by the host (Han and She, 2017). Different CRISPR-Cas systems have developed different expression system in order to produce crRNA. In type I and III, Cas6 alone or with other Cas proteins cleaves the pre-crRNA in repeats to form crRNAs. But in type II, trans-acting RNA (tracrRNA) binds to each repeats in the pre-crRNA to form a dual-RNA and this duplex RNA is cleaved by RNase III with the presence of Cas9 protein (Charpentier *et al*., 2015). This way the guide crRNA is produced which helps in cleavage activity.

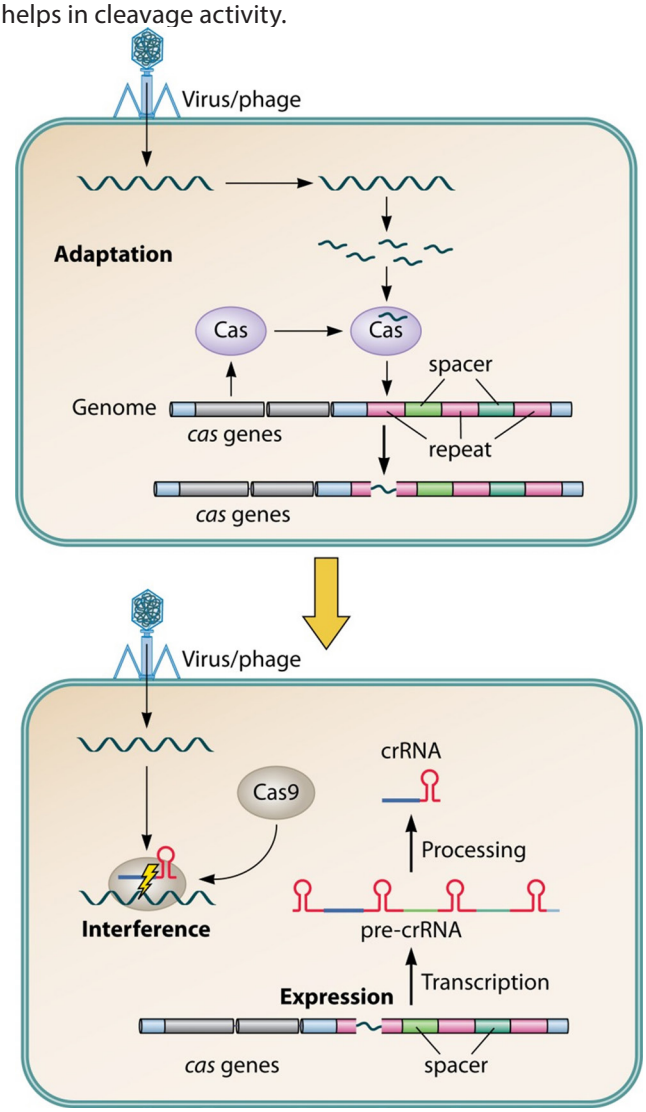

Fig. 1: Bacterial adaptive immune mechanism: (TOP) the invading viral genome is identified, cleaved by Cas protein, and adopted as spacer sequence as memory. (bottom) as the virus enters next time, the bacteria express pre-crRNA by transcription which is further processed into crRNA, which has homology with bacteriophage and interferes in the replication of virus (Ishino *et al.,* 2018).

Interference: The guide RNA formed from crRNA and tracrRNA along with Cas9 (type II, *Streptococcus pyogenes*) starts to screen the invading virus genome. Here the specificity of the guide RNA to viral sequence is established by complementarity between crRNA and target DNA, and also between Cas9 protein and protospacer adjacent motif (PAM) (Larson *et al*., 2013). PAM sequences are short 2–5 bp sequences contain recognition site for Cas9 protein, every CRISPR system or the host organism recognise different PAM sequence (Shah *et al*., 2013). Complementary of guide RNA and Cas9 protein with target DNA initiates the double strand break of target DNA with endonuclease activity of Cas9. Different bacteria have their own interference pattern using different Cas proteins, such as Cas12 and Cas13 in type V

and type VI CRISPR-Cas systems, respectively. In Class 1 multiple Cas proteins are involved in interference activity (Makarova *et al*., 2020).

The cleavage activity of Cas proteins guided by gRNA helps the host bacteria to eliminate the invading bacteriophage or any foreign DNA by obstructing the replication mechanism of the bacteriophages and act as adaptive immune system.

Applications

The ability of the CRISPR-Cas system to identify a specific sequence has been exploited to cut the DNA at a specific site and is used extensively to alter the genome and detect a specific sequence. The Class 2 CRISPR-Cas system's Cas9, Cas12 and Cas13 having single protein involved in formation of effector complex have been widely used in several applications (Ghorbani *et al*., 2021). Various applications are developed in this line to explore the beneficial genes of various organisms and manipulate for the betterment of human kind (Table 1, Fig. 2).

The production of these molecules to develop *in vitro* application is a vital component of CRISPR-Cas application. The gRNA involves crRNA and tracrRNA. crRNA has guide region and repeat region, whereas tracrRNA comprises of anti-repeat region and 3 stem-loop constructs (Allen *et al.,* 2021). There are few approaches to produce gRNAs such as chemical synthesis, *in vitro* transcription (IVT), and using gRNA-expressing DNA vectors (which are inserted into competent cells and subjected to transcription to produce gRNA) (Moon *et al*., 2019). Chemical synthesis of these molecules is cost effective and specific with pure quality of gRNA. The various applications of CRISPR-Cas system are discussed below:

Genomic editing: Genomic editing is central to various other applications of CRISPR, which is breakthrough in adopting the knowledge of bacterial adaptive immune system to alter various genes.

Among the various editing techniques followed earlier like Zinc Finger Nuclease (ZFN), Transcription activator-like effector nucleases (TALEN), CRISPR being an easier, cost effective tool for *in vitro* editing. Cas9 is extensively studied; crRNA-dependent endonuclease is having 2 nuclease domains, *i.e.,* HNH and RuvC. Nuclease cuts the target strand as well as non-target strand after finding the complementarity. If sgRNA finds the matching sequence then Cas9 binds to genomic locus next to PAM and makes double strand break (Zhang *et al*., 2020). The cell has two options, once the DNA strand is cut, the cell itself tries to repair with addition of nucleotide base pairs, this is referred as non-homologus end joining (NHEJ), which can cause addition or deletion of new genes leading to loss of function and structure. While the other option is to add the gene of our interest at the site of break, this is referred as homology directed repair (HDR) (Roy *et al*., 2018).

The dCas9 is an important innovation. Here the dCas9 refers to dead Cas9, which means there is introduction of mutation (at H840A and D10A, into HNH and RuvC nuclease domain) which makes it non-functional, but it retains its ability to bind to a specific sequence without cleaving it. This makes to act as manipulator in the transcription without editing the genes (Dominguez *et al*., 2016). Even the genomic loci can be visualised by tagging enhanced green fluorescent protein tagged to dCas9 and it is guided by gRNA specific to that loci (Chen *et al*., 2013)

Animal models: The genome editing can be applied to produce animal models whose genes are manipulated so that studies can be performed. A rabbit was produced with non-frame shift mutation of glucokinase gene with microinjection of Cas9 mRNA and gRNA. This animal mimicked the symptoms of hyperglycemia so that it can be used for the studies on diabetes drug screening and glucokinase role in pathophysiology (Song *et al*., 2020)

Gene therapy: The editing is used for genetic disorders to alter or delete or insert new gene. A CRISPR based gene deletion was performed for RHO gene as part of gene therapy for autosomal dominant retinitis pigmentosa (Tsai *et al.,* 2018). CRISPR based therapy is performed for sickle cell disease, β-thalassaemia and hemophilia B (Xu and Li, 2020), similarly it is applied to various other diseases.

Application in Disease Diagnostics

The CRISPR based approaches in disease diagnosis is a breakthrough in the molecular diagnostics with higher sensitivity, specificity and faster result (Table 1). Initially CRISPR based diagnosis were established for double-stranded DNA using Cas9 proteins. Here the Cas9 is guided with gRNA to cleave the dsDNA (sample) at the same time the reporter and quencher molecules are separated which results in the emittance of fluorescence and this is visualised with spectrophotometer (Kaminski *et al*., 2021).

The identification of the DNA can be without amplification and with amplification. The LOD for CRISPR based diagnostics is picomolar range (Ramachandran and Santiago, 2021). This makes the test highly sensitive and detects even minute quantity of DNA. Various amplification strategies are applied to improve the quantity of suspected DNA, *i.e.,* mainly involves isothermal amplification, such as Recombinase polymerase amplification (RPA), Loop mediated isothermal amplification (LAMP), Strand displacement amplification (SDA), Nucleic acid sequence-based amplification (NASBA) (Kaminski *et al*., 2021). The isothermal amplification does not require thermal cycler and other costlier setup. SHERLOCK stands for Specific High Sensitivity Enzymatic Reporter unlocking, which is a CRISPR based test with the ability to detect single copy of RNA of ZIKA virus. Here the RNA isolated is amplified using RT-RPA technique and the resulting DNA is transcribed into mRNA using T7 DNA dependent RNA Polymerase which is then detected by the Cas13a-crRNA and cleaved and is detected

Paper-based SHERLOCK (Gootenberg *et al*., 2017), makes it to be a point of care test with single copy sensitivity and even single base pair specificity.

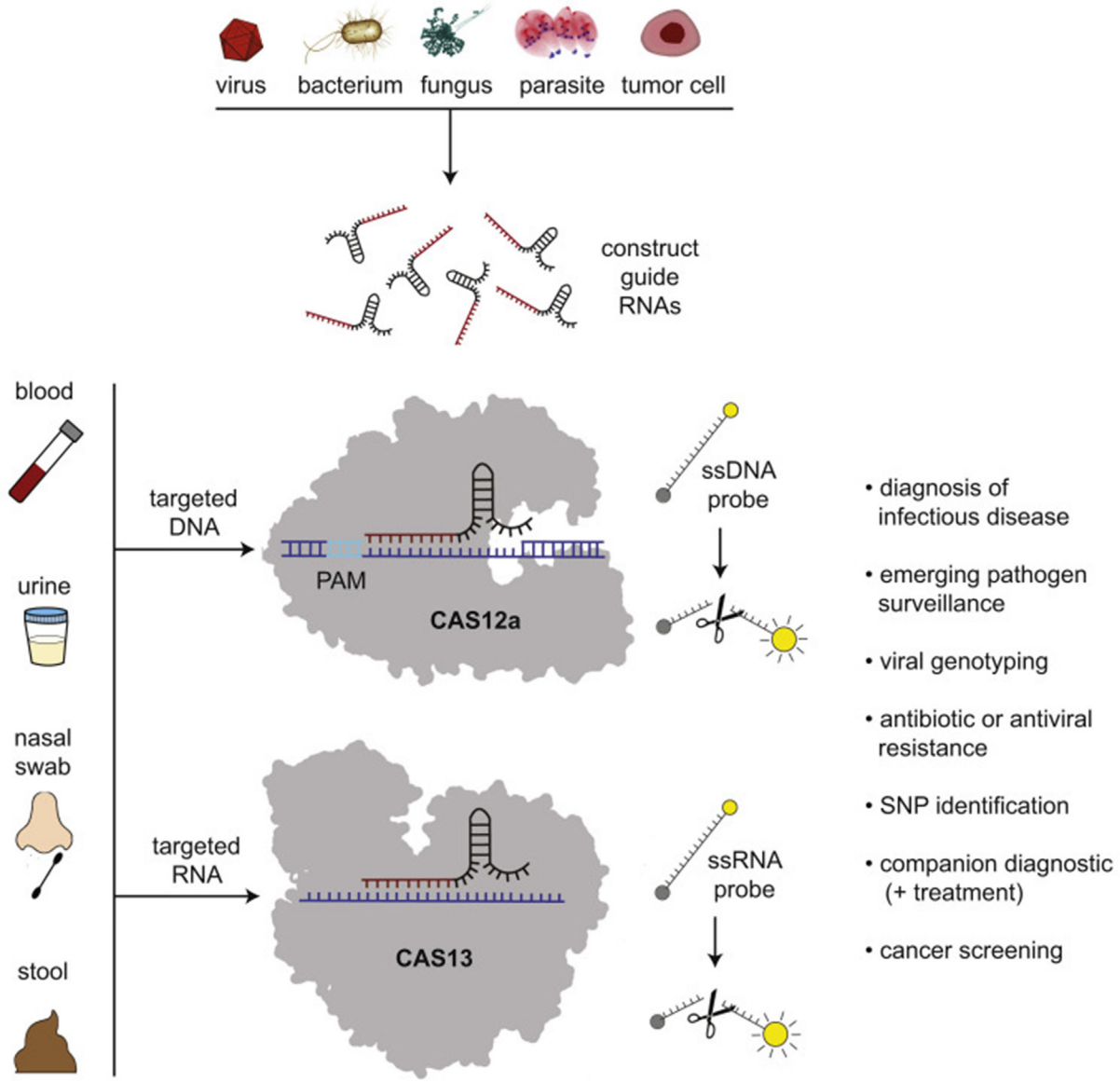

Fig. 2: gRNA is constructed targeting a specific region in the genome in pathogen, these gRNA are along with Cas12a or Cas13 bind to dsDNA and RNA respectively and initiate indiscriminate cleavage of fluorescent labelled probes producing detectable signal (Chiu, 2018).

DETECTR stands for DNA Endonuclease Targeted CRISPR Trans Reporter is another CRISPR based approach to detect the DNA of human papilloma virus. Here the DNA is isolated from the human anal swabs and DNA is amplified using RPA to produce sufficient copy numbers then the amplified product is added with LbCas12a-crRNA and fluorophore quencher (FQ)-labeled reporter molecule. Once the Cas12a binds to the complementary strand with the help of crRNA initiates robust, non-specific ssDNA trans-cleavage activity, which can be detected with the fluorescence (Chen *et al*., 2018).

Similar diagnostic approaches are even done for non-infectious diseases as well. The detection of miRNAs (noncoding RNAs), DNA methylation using CRISPR is novel idea. miRNA have potential role in the regulation of gene expression and can act as biomarkers and the presence of these molecules indicates a particular disease (Kim *et al*., 2021). This idea is extrapolated to develop cancer diagnostics detecting miRNA which are part of early tumour development, which acts as potential indicator and helpful to adopt preventive strategies (Shan *et al*., 2019). Detection of antimicrobial resistance genes in different microbes can be done at ease with CRISPR technology. The affecting pathogen can be isolated and the nucleic acids can be extracted and amplified. The resistant genes can be detected with various CRISPR-Cas systems (Wu *et al*., 2021).

Table 1: CRISPR based Zoonotic diagnostics

Sl.No	Name	Cas enzyme	Applications	LOD (moles/L)	References
1	CAS-EXPAR	Cas9	*Listeria monocytogenes* hemolysin (*hly*) gene	8.2×10^{-19}	(Huang *et al*., 2018)
2	Cas9nAR.	Cas9	*inv*A gene of Salmonella *typhimurium*, *uid*A gene of *E. coli*, *kat*G gene of *Mycobacterium smegmatis*, and *ind*A gene of *Salmonella erythraea*	1.66×10^{-19}	(Wang *et al*., 2019)
3	HOLMES	Cas12a	pseudorabies virus (PRV), RNA viruses (e.g., Japanese encephalitis virus (JEV)	1.0×10^{-17}	(Li *et al*., 2018)
4	APC-Cas	Cas13a	*Salmonella enteritidis*	1 to 10^5 CFU	(Shen *et al*., 2020)
5	SHERLOCK	Cas13a	Detection of viruses (ZIKV, DENV) and bacteria (*E. coli, K. pneumoniae, Pseudomonas aeruginosa, Mycobacterium tuberculosis, Staphylococcus aureus*);	2.1×10^{-18}	(Gootenberg *et al*., 2017)
6	CARMEN	Cas13	subtyping of influenza A strains and multiplexed identification of HIV drug-resistance mutations. a total of 169 human-associated viruses	9×10^{-19}	(Ackerman *et al*., 2020)

Scenario of CRISPR in India

The CRISPR technology in India is with increasing trend, as there is steady increase in publications from 2016 to 2019 (Roy Chowdhury and Gargate, 2021) , the Indian Govt. has further adopted the technique for diagnose and cure cases of sickle cell anaemia amongst the tribal population. The CRISPR is used as genome editing tool in most of the studies. A study was conducted to understand the VEGF A and FGF 2 signalling regulated by EGR on buffalo luteal cells, here EGR 1 was knocked out using CRISPR/Cas9-mediated gene editing technology and the effects were studied (Punetha *et al*., 2020). In agriculture sector it is used to develop disease resistant crop, CRISPR–Cas9 mediated genome editing is used to develop defense strategy against begomo viruses and cotton leaf curl viruses (Uniyal *et al*., 2019). Similarly, the application of CRISPR based editing is expanding in various fields veterinary, agriculture, biomedical research etc. The investment in biotechnology has to be boosted in India to reach greater heights and expand the economy. The application of CRISPR in the field of diagnosis remains greatly unexplored in India, the development of cost-effective diagnostic kits with high sensitivity and specificity can be used as a point of care diagnosis in India

Conclusions

CRISPR-Cas system of bacteria is used for wide range application to engineer the genes for benefit of the world. CRISPR having RNA guide makes it distinct from other protein-guided tools, RNA guide can be modified alone without affecting Cas proteins, this makes it precise, efficient and less difficult genome editing device. In the diagnosis the CRISPR has a potential scope due to its high sensitivity, specificity and potential to detect pathogenic genes in a cost-effective way. Since it does not require special equipment, it can be used as point of care diagnosis in any outbreak of diseases. This technology might empower the health services to address predominant public health issues of the country in epidemics and pandemics.

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