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CRISPR-based tools: Alternative methods for diagnosis of diseases

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Abstract
Recently developed nuclease-mediated genome editing technology, has stimulated interest in the generation and use of genome-edited livestock. Genome editing can be utilized to improve disease resistance, productivity as well as the generation of new biomedical models. Genome editing is a group of technologies which includes TALEN, ZFNs and CRISPR, that gives scientists the ability to change the DNA of an organism. Among them the CRISPR is the recent technology which has become an indispensable tool in biological research. CRISPR is the acronym for Clustered Regularly Interspaced Short Palindromic Repeats. The CRISPER technology uses the Cas9 and sgRNA for editing the target genome of interest. CRISPR-Cas9 is no longer just a gene-editing tool but can be used for other advanced applications which includes gene regulation, epigenetic editing, chromatin engineering, and imaging. CRISPR with the Cas system acts as an acquired immune mechanism in bacteria and archaea against viruses and bacteriophages. CRISPR array has repeats and spacers, repeats are palindromic sequences and each spacer is a virus-specific sequence Mechanism of bacterial adaptive immunity. Whenever any virus enters the bacteria for the first time, bacteria take up a part of the viral genome and adopt into the CRISPR array as a spacer sequence. When the virus enters the next time, bacteria produce gRNA which is complementary to the viral sequence and with help of Cas proteins cuts the foreign (viral) RNA and disrupts the viral replication thereby acting as a bacterial defence system.
The classes of the CRISPR-Cas system are defined by the nature of the ribonucleoprotein effector complex: class I systems are characterized by multiple effector proteins, and class 2 systems consist of a single crRNA-binding protein. For diagnostics, class 2 systems have primarily been applied for diagnostics, as these systems are simpler to reconstitute. They include enzymes with collateral activity. Which serve as the backbone of many CRISPR-based diagnostic assays.
Application of CRISPR involves Genomic editing, Genomic Regulation, Disease Diagnostics & treatment. Emerging therapeutic applications, Industrial and agricultural, and biological control. A diagnostic assay consists of a reaction of gRNA, Cas protein, reporter molecule and sample RNA. Here the gRNA along with Cas proteins screens the sample RNA. If there is a complementarity exists between gRNA and sample RNA, then Cas proteins start their cleavage activity and the reporter molecule emits fluorescence that can be detected with a fluorescence detection system, lateral flow device etc. The exploitation of the technology has been attempted in (HPV. ZIKA. tuberculosis, etc.). However, still remains an area of research for further extensive applications.

Keywords: CRISPR, diagnosis of diseases

Introduction
CRISPR and cas (CRISPR -associated protein) systems has revolutionized the gene editing field for research, biotechnology and potentially disease treatment in clinics. This technology possesses excellent features for manipulating genomes, such as easy design, low costs, rapid turnaround time and particularly its high accuracy and efficiency. Hence, CRISPR-Cas systems have multiple advantages and have overtaken the earlier -used gene-editing tools (Kaminski *et al*., 2021) [9]. Genome editing could be used to precisely introduce useful alleles (e.g. heat tolerance, disease resistance) and haplotypes into native locally-adapted cattle breeds, thereby helping to improve their productivity (Britt *et al*. 2018, Capper and Bauman, 2013) [4, 5]. As with earlier genetic engineering approaches, whether breeders will be able to employ genome editing in cattle genetic improvement programs will very much depend upon global decisions around the regulatory framework and governance of genome editing for food animals (Mottet *et al*., 2017) [10].

Tools of genome editing
Several nucleases have been successfully used for gene editing, including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and the clustered regularly

interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) protein 9 system. Over HR-based conventional gene targeting, all these nuclease-based gene editing tools enable site-directed genome engineering with tremendous advantages, including efficiency, low cost and simplicity, etc. A new era has arrived for genetic modifications, especially in large animals, for biological and biomedical investigation (Bevacqua *et al*., 2016) [2]. The first synthetically engineered, genome editing agents were ZFNs, which combine the binding module zinc finger protein (ZFP) with the restriction enzyme domain Fokl (an endogenous restrictive endonuclease from *Flavobacterium okeanokoites*). For me editing, a pair of ZFPs need to bind regions flanking the target locus to form a Fold dimer, which is necessary to induce double-strand breaks (DSB) (Zhao *et al*., 2019) [15].

Similarly, TALENS are also modular proteins that contain two domains: customizable DNA-binding domain (TALE) and a Fokl nuclease domain. Dimerized Fokt cuts TALE-binding DNA sequences, thereby producing DSBs in a similar way to ZVN TALEN-mediated gene editing was selected by the scientific society as one of the top 10 scientific breakthroughs in 2012, and both ZFNs and TALENs have been successfully used to generate genetically modified large animals. However, due to the extensive protein-DNA contacts of ZFNs and the highly repetitive nature of TALENs targeting of different sites in the genome by ZFNs and TALENS required the re-design or re-engineering of a new set of proteins. The difficulty in cloning and protein engineering ZFNs and TALENS partially prevented these tools from being broadly adopted by the scientific community. In this respect, CRISPR has revolutionized the field because it is as robust as, if not more so than the existing ools in terms of editing efficiency. More importantly, it is much simpler and more flexible to use. With significant technical barriers for ZFNs and TALENS, the CRISPR system has dominated the genome editing field since 2013 (Zhao *et al*., 2019) [15].

Discovery

The discovery of restriction enzymes that normally protect bacteria against phages in the late 1970 was a turning point that fueled the era of recombinant DNA technology. For the first time ever, scientists gained the ability to manipulate DNA in test tubes. Although such efforts drove a number of discoveries in molecular biology and genetics, the ability to precisely alter DNA in living eukaryotic cells came a few decades later. (Ishino *et al.,* 2018) [7]. For several years, scientists have been using 'gene targeting' to introduce new changes into a specific site in the genome by removing or adding single bases or whole genes. Furthermore, researchers have used technologies derived from the prokaryotic immune system. Systems involving the clustered regularly interspaced short palindromic repeat (CRISPR) and its associated proteins (Cas) have become the most reliable tools for gene editing. The idea of the CRISPR-Cas technique has been adapted from the bacterial immune system. The CRISPR-Cas9 system has been widely adopted all over the world and successfully applied to target essential genes in different organisms and cell lines, including bacteria, zebrafish, monkeys, rabbits, mice and even humans.

The first CRISPRS were detected 30 years ago by one of the authors of this review (Ishino *et al*., 2018) [7] in Escherichia coli in the course of the analysis of the gene responsible for isozyme conversion of alkaline phosphatase. They found unusual repeated sequences and due to the lack of sufficient DNA sequence data, especially for mobile genetic elements. The actual function of this unique sequence remained enigmatic until the mid-2000s. In 1993, CRISPRS were for the first time observed in archaea, specifically in Haloferax mediterranei, and subsequently detected in an increasing number of bacterial and archaeal genomes, since life science moved into the genomic era. Conservation of these sequences in two of the three domains of life was critical for an appreciation of their importance. In the early 2000s, the discovery of sequence similarity between the spacer regions of CRISPRS and sequences of bacteriophages, archaeal viruses, and plasmids finally shed light on the function of CRISPR as an immune system.

This dramatic discovery by Mojica and others was grossly underappreciated at that time and was independently published in 2005 by three research groups. In parallel, several genes previously proposed to encode DNA repair proteins specific for hyperthermophilic archaea were identified as being strictly associated with CRISPR and were designated cas (CRISPR-associated) genes. Comparative genomic analyses thus suggested that CRISPR and Cas proteins (the cas gene products) actually work together and constitute an acquired immunity system to protect prokaryotic cells against invading viruses and plasmids, analogous to the eukaryotic RNA interference (RNAi) system.

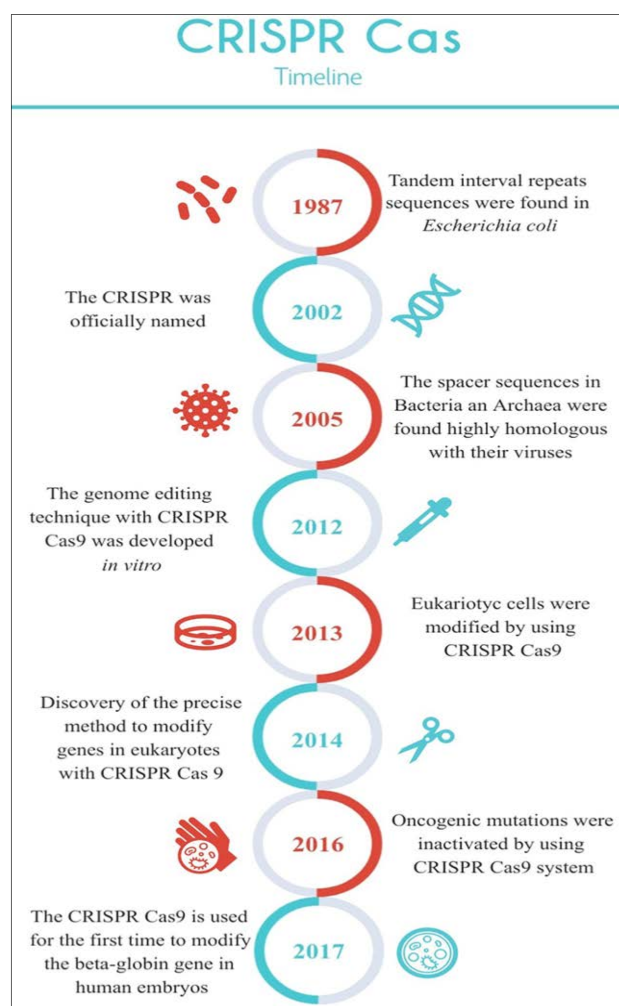

Fig 1: CRISPR Cas Timeline

Classification

Due to differences of core Cas proteins, CRISPR-Cas systems have been categorized into two classes (1 and 2) and

subdivided into six types (I-VI) with diverse subtypes. (Ishino *et al*., 2018) [7] The class 1 CRISPR-Cas system, which functions through a multi-Cas protein complex, includes types I, III, and IV, employing representative endonucleases of Cas3, Cas 10, and DinG, respectively. The class 2 CRISPR-Cas system, which employs single Cas protein, includes types II, V. and VI, to cleave RNA-guided genetic codes with Cas9, Cas12-Cas14, and Cas13, respectively. Types I, II, and V systems are shown to specifically target DNA, and type III targets both RNA and DNA; type VI can only edit RNA. The function and mechanisms of type IV system remain largely unknown (Palaz *et al*., 2021) [12].

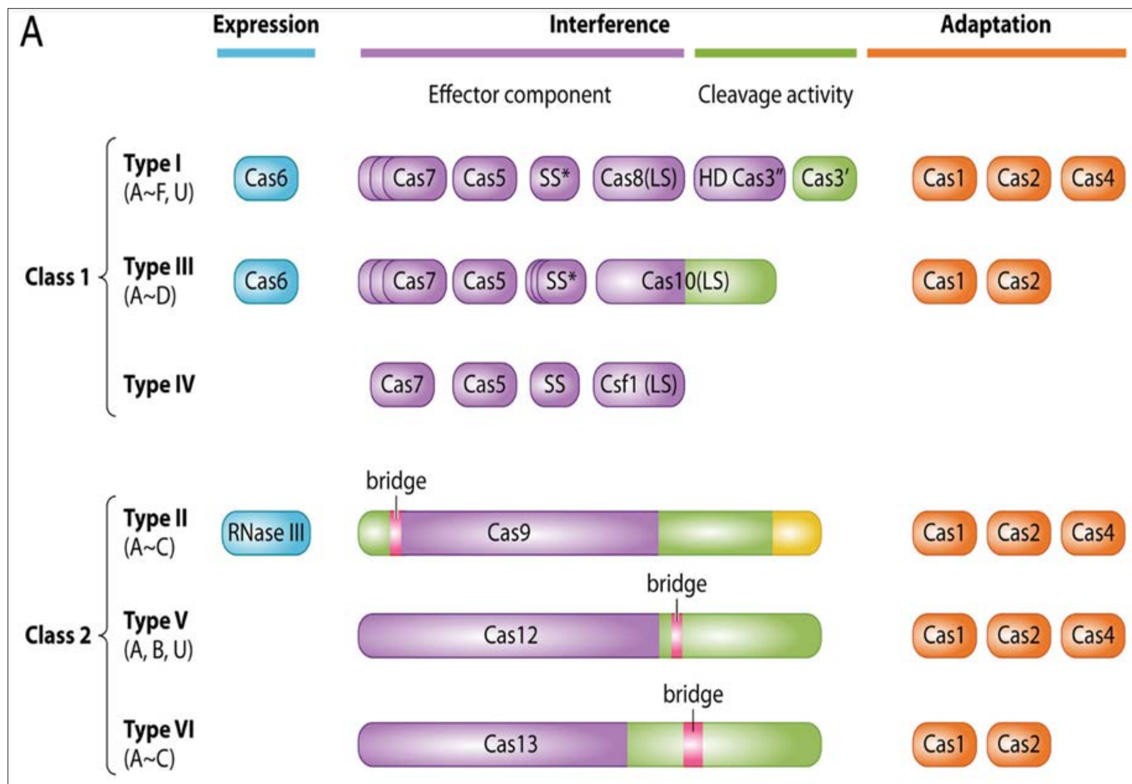

Fig 2: Classification of CRISPR-Cas system

Scenario of CRISPR in INDIA

- Institute of Genomics and Integrative Biology (IGI), Delhi- is presently working to edit the gene responsible for sickle cell anemia.
- Junagadh Agricultural University (JAU) is on its way to produce Cholested-Bree ol using CRISPR technology
- National Agri-Food Biotechnology Institute, Mohali. Working to improve the nutritional quality of Rasthali banana variety.
- India's biotech market presently shares 2% of the global biotech market so there is abundant scope for us to improve
- At IVRI, Gene editing of the T. evansi PFR1 gene has been performed to alter the flagellar proteins of T. evansi which can be possible ways to produce immunogen without pathogenicity. Which would be of great help in the production of vaccines too.

Recent advances in genome editing using CRISPR/CAS

The CRISPR/Cas9 system has been adapted as an efficient genome editing tool in laboratory animals such as mice, rats, zebrafish and pigs. It was reported that CRISPR/Cas9 mediated approach can efficiently induce monoallelic and biallelic gene knockout in goat primary fibroblasts. Four genes were disrupted simultaneously in goat fibroblasts by CRISPR/Cas9- mediated genome editing. The single-gene knockout fibroblasts were successfully used for somatic cell nuclear transfer (SCNT) and resulted in live-born goats harbouring biallelic mutations. The CRISPR/Cas9 system represents a highly effective and facile platform for targeted editing of large animal genomes, which can be broadly applied to both biomedical and agricultural applications. Cas9/gRNAs could induce precise mutations with efficiency of 9%-70% in goat primary fibroblasts. A single co-transfection of pooled Cas9/gRNAs enabled isolation of cell colonies carrying simultaneous disruption of four genes with high efficiency. The Cas9/RNA-modified fibroblasts were subjected to nuclear reprogramming by somatic cell nuclear transfer, resulting in live-born goats carrying single gene mutation (Ni *et al*., 2014) [11].

The double-muscled sheep caused by natural loss-of-function mutations of MSTN have very strong skeletal muscle. In a study, it was demonstrated the successful generation of MSTN mutant sheep via specific targeting of an exon 1 site using Cas9 technology. The MSIN knockout sheep in the study had increased muscle significantly just like double-muscled phenotype. This study suggested that the direct injection of Cas9: sgRNA into zygotes could be widely used to create gene knockouts in large domestic animals. Notably, on the basis of the findings, sheep can be added to the growing list of species for which genome editing is now practical. The generation of MSTN mutant sheep has implications for the genetic improvement of local sheep varieties, and also for the usage of sheep as a model for large animal medical research (Kalds *et al*., 2019) [8].

Disease resistance

Bovine tuberculosis, which is caused by Mycobacterium

bovis, is becoming a serious threat to the agricultural economy and global public health (transmission from cattle to humans). Currently, no effective programs exist to eliminate or control bovine tuberculosis.

One gene of interest is the natural resistance-associated macrophage protein-1 pe (NRAMPI), which is also known as the solute carrier family 11A member gene (SL.CIIAI). The gene has been found to be associated with innate resistance to intracellular pathogens such as Mycobacterium, Leishmania, Salmonella and Brucella, and the resistance is suspected to be induced by multiple proinflammatory responses. Indeed, transgenic cows with a site specific NRAMPI insertion confirmed the function of NRAMP in providing resistance to tuberculosis (Alexandratos *et al*., 2012) [1].

Foot-and-mouth disease virus (FMDV) is also another economically devastating viral disease facing the swine industry worldwide. Transgenic pigs were generated that constitutively expressed FMDV-specific short interfering RNAs derived from small hairpin RNAs and transgenic pigs exposed to the virus displayed no clinical signs of viral infection when compared with wild-type pigs, offering another example of genetic engineering for disease resistance.

Animal welfare

Physical dehorning of cattle is used to protect animals and producers from accidental injury, but is costly and painful for the animals. Genetic analyses have identified variants that are associated with cattle hornlessness (ie, 'polled'), a trait that is common in beef but rare in dairy breeds. Fewer beef cattle than dairy cattle need to be dehorned because the dominant POLLED locus is nearly fixed in beef cattle, such as Angus. Dairy breeds, such as Holstein. Have a much lower frequency of POLLED with only a small number of sires (6%) producing commercially available POLLED semen. Thus, a candidate 'polled allele was introgressed into dairy cattle using TALEN-mediated genome modification and reproductive cloning Hornless dairy cattle were obtained, providing evidence for genetic causation and a means to introduce polled into livestock with the potential to improve the welfare of millions of cattle without crossing. (Yao *et al*., 2016) [14].

Applications of CRISPR with special reference to Diagnostics

Application of CRISPR involves Genomic editing. Genomic Regulation, Disease Diagnostics & treatment, Emerging therapeutic applications, Industrial and agricultural, and biological control.

CRISPR has great potential to cure and treat monogenic diseases and has already been applied within animal models for gene studying. The applications for different therapeutic methods to create a genetic cure are limited by the fact that disease causing mutations in early embryos (germline editing) are significantly easier to correct than in somatic cells, partly since the modification has to be delivered to trillions of cells (Binnie *et al*., 2021) [3]. CRISPR successfully has been editing genes in non-viable human embryos, with spread results concerning safety and mosaic embryo.

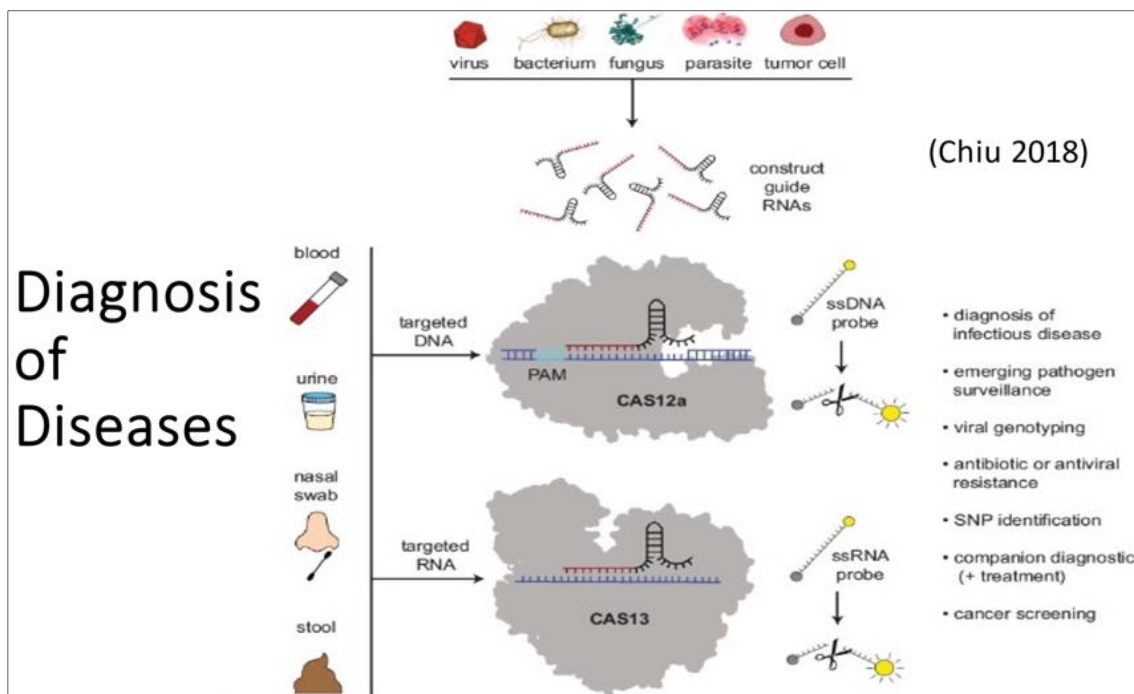

Fig 3: Diagnosis of Diseases

The multiplex characteristics of CRISPR have great potential to treat cancer, a disease killing one out of six people worldwide. The focus to beat cancer today, lies in the understanding of carcinogenesis since it is caused by the interplay of many genes. The limitations are, for instance, finding the mutations causing tumor growth in a large library of different mutations and tangled connections. In this aspect, the CRISPR-Cas system can be retooled to find important target sites and describe gene functions with a greater speed by utilizing multiplexing. Within immunotherapy, the CRISPR molecules can be programmed to act as GPS coordinates and recognize cancer cells by reprogramming T-cells to locate and differentiate tumor cells from healthy cells (Kaminski *et al*., 2021) [9].

Further, CRISPR-Cas systems have shown great potential to be a ground-breaking tool within diagnostics. The novel and ongoing pandemic from SARS-CoV-2 is dependent on fast, cheap, and correct testing to control the spread of the

coronavirus and limit the mortality rate (Palaz *et al*., 2021) [12]. The CRISPR diagnostic methods may stop the rapid spread of the virus earlier since the results do not take hours to get back. A test based on the CRISPR-technology gives results back within one hour, compared with other time-consuming methods. RT-PCR is a frequently applied test used today to identify the virus particles but has some considerable limitations and setbacks. RT-PCR is a time-consuming process, requires laborious procedures and is also sensitive to deamination (Srivastava *et al*., 2020) [13].

Several labs have through CRISPR developed tests to identify invasion of coronaviruses, like the SHERLOCK-test from Broad institute (Feng Zhang) and DETECTR Biosciences.
The SHERLOCK test utilizes the Cas13 pride cuts in RNAs, breaking it into smaller pieces in a p RNA cleavages can be manipulated or marked to be age give distinct symbols on a paper strip, similar to the signs based test produced by Sherlock Biosciences was on t May 26 SPD by the FDA to use for detection of corona-cases (Gootenberg *et al*., 2017) [6].

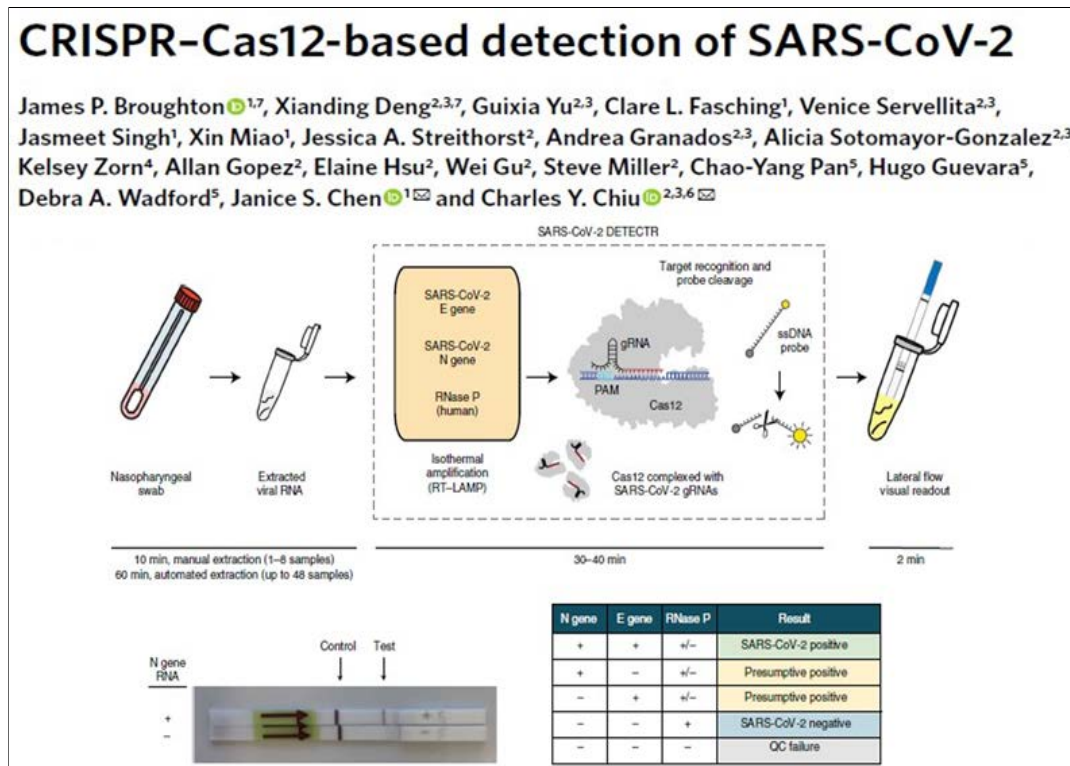

Fig 4: CRISPR-Cas 12-based detection of SARS- CoV-2

We are entering a new era, one in which gene editing will become progressively more straight forward, and more essential to animal welfare and livestock productivity Beneficial es that would otherwise be lost in conventional breeding could be conserved using the novel genome editing tools, and also result in reduced cost and a shortened timeframe for generating the desired mutant animals. Precision editing in the endogenous genome, without introducing foreign DNA, could become a new breeding technology to produce genetically modified organisms for human consumption. By combining genomic selection and genome editing, we can add highly valuable mutations, even those that would be outside of the available breeding population, onto the best genetic backgrounds. Genome editing is not the sole answer, but when combined with genomic selection and assisted reproductive technologies, could transform current livestock improvement strategies (Zhao *et al*., 2019) [15].
In the next decades, it may be that every animal brought to state fairs by 4-H animals contains his or her own personal edits, unlocking the creative potential of the next generation in the way that microelectronics or the internet fascinated previous generations of livestock's TALEN and CRISPR/Cas9 enable targeted edits in a way never before possible, and succeeding improved generations of these site-specific nucleases will only increase efficiency and specificity. Right now, these are made into live animals through somatic cell nuclear transfer or zygote injection, but one can imagine a not-too distant future in which cells are directly transformed into spermatozoa in a dish, making large animal genome editing accessible to a wider population.

Conclusions

The CRISPR-based technologies will undoubtedly continue to transform basic as well as clinical and biotechnological research. Genome editing in large animals has tremendous practical applications. Crispr/Cas9 could be harnessed for direct modification of somatic tissue, obviating the need for embryonic manipulation as well as enabling therapeutic use for gene therapy.

- CRISPR array acts in the bacterial adaptive immune system
- Cas proteins are CRISPR-associated proteins, help in adaptation, expression, interference activity CRISPR sequences have been found in the prokaryotic genome
- CRISPR array has spacers and repeats Class 2 systems are used for diagnostic approaches
- Point of care diagnosis in resource-poor settings
- Investment in CRISPR research has to be conducted in India to develop newer diagnostics
- Development of immunogens in vaccine production

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