OMMEGA Publishers Journal of Veterinary Science and Animal Welfare

OPEN ACCESS

Research article

ISSN: 2690-0939

Fesseha, H., et al. | J Vet Sci Animal Welf 4(1): 1-9.

Application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in Veterinary Science: A Review

Haben Fesseha[1*], Takuma Fetene[2]

[1]School of Veterinary Medicine, Wolaita Sodo University, Ethiopia, PO Box 138, Wolaita Sodo, Ethiopia
[2]College of Veterinary Science, DVM, Mekelle University, Ethiopia, PO Box 2084, Mekelle, Ethiopia

***Corresponding author:** Haben Fesseha, School of Veterinary Medicine, DVM, MVSc, Wolaita Sodo University, Ethiopia, PO Box 138, Wolaita Sodo, Ethiopia, Tel : +251972851714; E-mail: tseyon.h@gmail.com

Abstract

Genome engineering has shown tremendous promise in many aspects of disease prevention and therapy, vaccine development, animal welfare, and food security. However, the practice of genome editing has long been limited to countries and institutions with a better facility and expertise. More recently, the advent of a nuclease-based genome editing technique called CRISPR / Cas9 shades a great light in the genome engineering field. It is based on the principle that the cas9 endonuclease can be guided by a guide RNA to precisely cut the target in the genome. It recently became the most favorite technique among scientists because of its relative specificity, ease of handling and cheaper cost. It holds a great promise to scale up animal and crop production in the near future. Although some ethical concerns are being raised, it is important for countries like Ethiopia; to develop, validate and benefit from the technology.

Keywords: CRISPR; CRISPR / Cas; Genome editing; CRISPR Applications; Animal models; Gene therapy

Introduction

Knowledge of the principles of heredity is basic to our fundamental understanding of biological processes. The concept of modern biotechnology including genetic engineering has been conceived and advanced after Gregor Mendel demonstrated that traits can be inherited from generation to generation[1].

Genetic engineering is often thought to be rather emotive or even trivial, yet it is probably the label that most people would recognize. However, there are several other terms that can be used to describe the technology, including gene manipulation, gene cloning, recombinant DNA technology, genetic modification[2]. The premise on which the technology is based is that genetic information, encoded by DNA and arranged in the form of genes, is a resource that can be manipulated in various ways to achieve certain goals in both pure and applied science and medicine[3,4].

In genetic engineering and its classification, DNA, mRNA, transcription, translation, mutation, and mutagenesis some basic terms that should be understood. Hence, DNA molecule is a double-strand helix that is wrapped around proteins to form chromosomes in the nucleus of each cell[4,5]. The central dogma of molecular biology describes DNA makes mRNA and mRNA in-turn makes proteins. The genetic code describes the relationship between the sequence of base pairs in a gene and the corresponding amino acid sequence that it encodes[6,7].

The development and function of an organism is controlled by the codes in the DNA. A random change in the DNA sequence is called a mutation. Mutations can lead to changes in the structure, function or amount of an encoded protein. Because a change in the DNA sequence affects all copies of the encoded protein, mutations can be particularly damaging to a cell or organism or it can be used to modify the inherent characteristics of an organism by editing its gene composition. Changes in DNA sequence can occur randomly or can be induced deliberately in the laboratory. Mutagenesis in the laboratory is an important technique whereby DNA mutations are deliberately engineered to produce mutant genes, proteins, strains of bacteria, or other genetically modified organisms[6,8,9].

Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) are technologies have

Received date: December 10, 2019
Accepted date: January 31, 2020
Published date: February 4, 2020

Citation: Fesseha, H., et al. Application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in Veterinary Science: A Review. (2020) J Vet Sci Animal Welf 4(1): 1-9.

been developed to facilitate programmed mutagenesis[10]. However, the complexity and lower efficiency of these methods limit their utility. Recently, the type II bacterial CRISPR (clustered regularly interspaced short palindromic repeats) / Cas (CRISPR-associated) system has been developed as an easy, inexpensive, efficient and versatile technology for genome editing in eukaryotic cells and whole organisms[11,12]. Although CRISPR / Cas9 is the most favorite new technology, the practical application of this latest method is not fully started throughout the world; particularly in developing countries. Consequently, the review tries to address the current application and understandings of genome editing technology with an emphasis on CRISPR / Cas9 especially in the context of veterinary medicine.

Historical perspectives

In 1987, Yoshizumi Ishino discovered unusual DNA repeats of unknown function in the genome of the bacteria Escherichia coli. Later in 2000, Mojica identified these same types of repeats in other microbes and termed these to be Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). Later work uncovered similar repeat structures in a wide range of bacteria and archaea. It was initially unclear what role these conserved repeats might play in the prokaryotic genome[13-15].

Table 1: Timeline of CRISPR/Cas9 development.

Year	Advancements and Developments
1987	First report of CRISPR repeats
2000	Recognition that CRISPR families are present throughout prokaryotes
2002	Coined “CRISPR” name defined Cas genes
2005	Identified foreign origin of spacers proposed adaptive immunity function
2007	First experimental evidence for CRISPR adaptive immunity
2008	CRISPR acts upon DNA targets Spacers are converted into crRNAs that act as small guide RNAs
2009	Type III-B cmr CRISPRcomplexes cleave RNA
2010	Cas9 is guided by spacers sequences and cleaves target DNA via DNA double-strand breaks (DSBs)
2011	tracrRNA forms a duplex structure with crRNA in association with Cas9 type II CRISPR systems are modular and can be heterologously expressed in other organisms
2012	Invitro characterization of DNA targeting by Cas9
2013	First demonstration of Cas9 genome engineering in eukaryotic cells
2014	Genome-wide functional screening with cas9 Crystal structure of apo-Cas9 Crystal structure of cas9 in complex with guide RNA and target DNA

Source:[14,16].

Genome Editing

Genome editing emerged in the laboratory of Paul Berg in 1972 in the form of a recombinant DNA technology, when scientists combined the E. coli genome with the genes of a bacteriophage and the SV40 virus[17,18]. Since then, this science has achieved tremendous success. One of the challenges of genetic engineering is the ability to select a specific gene and alter it, or add another gene or delete a gene. A key step in all of these is the ability to cut and paste at specific sites, at very specific sites. Now that one can read a gene in detail and when one knows what the desired result should be, then the cut and paste side is critical. Pasting is somewhat well known, especially if we have cut at the right location[19].

Types of genome editing techniques

Technologies for making and manipulating DNA have enabled advances in biology ever since the discovery of the DNA double helix. But introducing site-specific modifications in the genomes of cells and organisms remained to be a challenge. Based on the use of gene modifying agents, genome editing techniques can be classified as either non-nuclease based or nuclease based techniques[20-22].

Non-nuclease-based genome editing

This technique implements the use of physical agents such as; fast neutron, Ultraviolet (UV) and x-ray radiation, or chemical agents such as N-methyl-N-nitrosourea (MNU), or ethyl methanesulfonate (EMS)[23]. Among these compounds, EMS has become one of the most effective, reliable, powerful and frequently used chemical mutagens. EMS mainly induces Cytosine (C)–to-Thymine (T) substitutions resulting in Cytosine/Guanine (C/G) to Thymine /Adenine (T/A) transitions and at a low frequency, EMS generates G/C to C/G or G/C to T/A transversions through 7-ethylguanine hydrolysis or A/T to G/C transitions through 3-ethyladenine pairing errors[24]. The major challenge in chemical mutagenesis stems from the large number of mutations induced per genome, which requires the implementation of tedious genetic mapping techniques to locate the causative mutation to a defined genomic region[25].

Nuclease based genome editing

Targeted genome editing is a broadly applicable approach for efficiently modifying essentially any sequence of interest in living cells or organisms[26]. This technology relies on the use of engineered nucleases; artificial proteins composed of a customizable sequence-specific DNA-binding domain fused to a nuclease that cleaves DNA in a non-sequence-specific manner. These targetable nucleases are used to induce double-strand breaks (DSBs) into specific DNA sites, which are then repaired by mechanisms that can be exploited to create sequence alterations at the cleavage site. Nuclease-mediated genome editing enables genetic studies that were previously difficult or impossible to perform [27,28].

Early approaches relied on the principle of site-specific recognition of DNA sequences by oligonucleotides, small molecules, or self-splicing introns[29]. In the last decade, the pace of genome engineering genome editing has gained great momentum. Several new approaches have been developed including the use of the site-directed ZFNs, TALENs using the principles of DNA-protein recognition was developed. However, difficulties of protein design, synthesis, and validation remained a barrier to the widespread adoption of these engineered nucleases for routine use11, 12. More recently, the two-component RNA-guided endonucleases (RGENs) mediated technique has been developed and is being widely used in biomedical research. This technique is known as the CRISPR / Cas system. It changes the landscape of genome editing in a wide variety of organisms[30,31].

Zinc finger nucleases (ZFNs)

Zinc-finger domain is among the most common types of DNA-binding motifs found in eukaryotes and represents one of the most frequently encoded protein domains in the eukaryotic genome. An individual zinc-finger consists of approximately 30 amino acids in a conserved helical configuration. Several amino acids on the surface of the α-helix typically contact 3 base-pair in the major groove of DNA, with varying levels of selectivity[32]. The modular structure of zinc-finger proteins has made them an attractive framework for the design of custom DNA-binding proteins[33].

ZFNs consist of a customized DNA binding domain and a DNA cleavage domain. The DNA-binding domain contains a sequential fusion of multiple zinc finger domains, which bind to specific DNA target sequences. Each zinc finger domain binds a three base pair sequence of DNA. The DNA cleavage domain employs the type IIs restriction endonuclease FokI. Because the double-stranded cleavage activity of FokI requires dimerization, a pair of ZFNs that bind on opposite sides of the target site is used. To date, there are many reports of ZFN-based genome editing of a variety of organisms[34]. Despite the advances of ZFN-based methods in basic and translational research, ZFN technology has several limitations. First, because zinc finger domains have a higher affinity for G-rich sequences, there are several three-base nucleotide sequences that do not have associated zinc finger binding domains, which limit the robustness and specificity of ZFN-mediated cleavage. Second, off-target cleavage by ZFNs can alter the function of unknown genes, introduce oncogenic translocations, and result in elevated cytotoxicity. Finally, the generation of functional ZFNs requires a significant amount of expertise in protein engineering.

Transcriptional activator-like effector nucleases (TALENs)

Similar to ZFNs, TALENs are chimeric proteins comprised of a programmable DNA-binding domain fused to the Fok1 nuclease domain. TALEs are naturally occurring proteins that are secreted by the bacteria Xanthomonas and bind to sequences in the host plant genome, activating transcription. The TALE DNA-binding domain is composed of multiple repeats, each of which is 33-35 amino acids long[35]. Each repeat recognizes a single nucleotide in the target DNA sequence[36,37].

Nucleotide specificity is conferred by a two-amino-acid hypervariable region present in each repeat. Sequence-specific TALENs are generated by modifying the two residues in the hypervariable region and concatenating multiple TALE repeats together. Because the TALE DNA-binding domain is fused to Fok1, TALENs, like ZFNs, must also be used as dimers to generate DSBs[39,40].

Clustered regularly interspaced palindromic repeats (CRISPR / CAS9)

CRISPR / Cas9-mediated genome editing depends on the generation of double-strand break (DSB) and subsequent cellular DNA repair process. In the endogenous CRISPR / Cas9 system, mature crRNA is combined with trans activating crRNA (tracrRNA) to form a tracrRNA: crRNA complex that guides Cas9 to a target site[41,42]. The development, advancement and current understanding of the CRISPR/Cas9 technique are discussed in Table 2, the emphasis given on its application in veterinary research.

Table 2: Comparison of engineered nucleases for targeted gene editing.

	Features	Advantages	Limitations
ZFNs	FoKl endonuclease fused to zinc fingers(ZF) which interact with target DNA 4-6 ZF motifs required to confer specificity Each ZF motif recognizes 3 base pairs of DNA Single strand cleavage	High specify Low immunity Currently being used in clinical trials	Non-modular; difficult and laborious engineering process Difficult to predict cytotoxic off-target effects Some nucleotide triplets do not have corresponding ZF Pair of ZFNs required to target specific DNA sequence
TALENs	FoKl endonucleases fused to TALE domains which interact with target DNA 1 TALE domain recognizes 1 DNA base pair Single strand cleavage	Modulator assembly, more straightforward assembly Fusing multiple TALE domains do not affect binding specificity Simpler targeting efficiency with less cytotoxic effects	5’ base of TALEN target site must be a thymine Off-target effects TALEN binding is negatively impacted by DNA methylation
CRISPR/Cas9	Cas9 endonuclease derived from S.Pyogens recruited to DNA via sgRNA Site specificity results from 20 base pairs of sgRNA interacting with target DNA Double strand break	Easy to generate via simple molecular cloning techniques Higher target efficiency Ability to multiplex for targeting of multiple genes	Requirement for a PAM sequence adjacent to the target site Higher off-target effects Binding efficiency impacted by chromatin accessibility

Source:

Application of CRISPR / CAS9 in Veterinary Science

Basic molecular mechanism of CRISPR / Cas9 in bacterial immunity

CRISPR / Cas systems are diverse in widespread prokaryotic systems found in almost every archaeal and 40 % of bacterial species sequenced. Based on the presence of signature Cas proteins, CRISPR / Cas systems are classified into three types: type I, II, and III for the presence of Cas3, Cas9, and Cas10, respectively. The three types were further divided into more complex subtypes depending on the presence of additional signature proteins. However, the type II CRISPR / Cas system containing the

Cas9 protein is commonly used for eukaryotic genome editing nowadays[42,43].

Although out populated and preyed upon by abundant and ubiquitous viruses, microbes routinely survive, persist, and occasionally thrive in hostile and competitive environments. The constant exposure to exogenous DNA via transduction, conjugation, and transformation have forced microbes to establish an array of defense mechanisms that allow the cell to recognize and distinguish incoming "foreign" DNA, from "self " DNA and to survive exposure to invasive elements Bacteria defend against invading DNAs with an adaptive immune strategy using a class of RNA-guided nucleases[44-46]. The RNAs are generated from a CRISPR in bacterial genomes. The CRISPR-associated (Cas) nucleases are then guided to foreign DNA targets by these CRISPR RNAs (crRNAs) by the recognition of protospacer adjacent motifs (PAMs) in the foreign DNA sequence. After complementary DNA-RNA base paring, the Cas nuclease cleaves the target DNA by forming site-specific DSB.

Therefore, the defensive CRISPR/Cas9 system from *Streptococcus pyogenes* (*S. pyogenes*) has been most studied and provides researchers with a promising tool for specific genome editing, as in vivo DSB will be repaired mainly through non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways, introducing insertions or deletions. The most explored CRISPR/Cas system for genome editing to date employs the *S. pyogenes* Cas9 nuclease, which can recognize the PAM sequence NGG (N is A, T, C or G)[47].

Bacteria have developed a technique where they can recognize a foreign viral DNA segment and then "attack" is with an enzyme and a targeted RNA segment that results in the foreign DNA being broken and becoming ineffective. This bacterial process effectively kills the DNA of the invader, stops its reproduction and induces autophagy.

Adaptive immunity in this bacteria and archaea occurs in three stages:

- o insertion of a short sequence of the invading DNA as a spacer sequence into the CRISPR array (adaptation phase);
- o transcription of precursor crRNA (pre-crRNA) that undergoes maturation to generate individual crRNAs, each composed of a repeat portion and an invader targeting spacer portion(biogenesis phase); and c
- o rRNA-directed cleavage of foreign nucleic acid by Cas proteins at sites complementary to the crRNA spacer sequence(interference phase) 48. The protospacer adjacent motif (PAM), a short sequence motif adjacent to the crRNA-targeted sequence on the invading DNA, plays an essential role in the stages of adaptation and interference. The type II system requires only a single protein for RNA-guided DNA recognition and cleavage, a property that proved to be extremely useful for genome engineering applications.

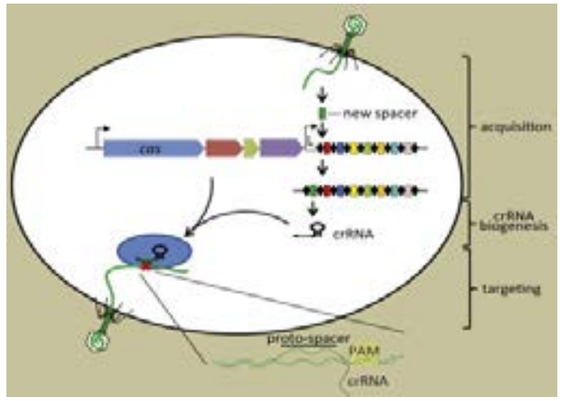

Figure 1: The three Stages of CRISPR Immunity.

Source: [49]

CRISPR loci contain clusters of repeats (black diamonds) and spacers (colored boxes) that are flanked by a "leader" sequence (L) and CRISPR-associated (*Cas*) genes. During adaptation, new spacers derived from the genome of the invading virus are incorporated into the CRISPR array by an unknown mechanism. The synthesis of a new repeat is also required. During crRNA biogenesis, a CRISPR precursor transcript is processed by Cas endoribonucleases within repeat sequences to generate small crRNAs. During targeting, the match between the crRNA spacer and target sequences (complementary protospacer) specifies the nucleolytic cleavage (red cross) of the invading nucleic acid.

Development of CRISPR / Cas9 as genome editing tool

In 2012 Jennifer Doudna and Emmanuelle Charpentier first utilized CRISPR/Cas9 to induce specific DSBs. Later in 2013, Cong and colleagues designed two different type II CRISPR/Cas systems and demonstrated that Cas9 nucleases can induce precise cleavages at specific sites under the guidance of short RNAs in human and mouse cells.

The type II system genomic CRISPR locus includes the tracrRNA gene, Cas gene, and CRISPR repeat-spacer array, which are transcribed into tracrRNA, Cas9 proteins and pre-crRNA, respectively. The Cas9: RNA complex randomly searches DNA sequences and rapidly binds to the PAM sequence. After binding to the PAM sequence (usually NGG motif for SpCas9, sometimes NAG motif), the Cas9: RNA complex interrogate the flanking DNA sequences for gRNA complementarity. At the target sites that match the tracrRNA: crRNA complex and are adjacent to PAMs, the HNH nuclease domain of Cas9 cut the strand that binds to crRNA, and the RuvC-like domain cleaves the opposite DNA strand to produce DSBs at the specific sites[50].

To avoid genotoxicity and achieve physiological levels of transgene expression, gene-specific genome editing or site-specific gene addition is preferable to the random integration of expression cassettes, which could result in insertional mutagenesis and cancer as well as silencing of transgene expression[51]. CRISPR is a tool that does just that specificity, it is a very accurate, fast, and low-cost gene cutting tool.

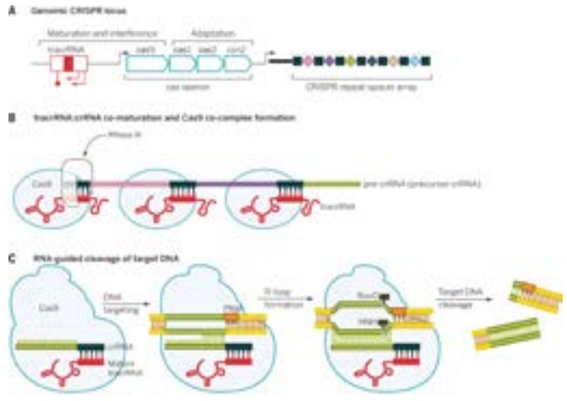

Figure 2: Biology of the type II-A CRISPR-Cas system.

Source:

The type II-A system from *S. pyogenes* is shown as an example. (A) The Cas gene operon with tracrRNA and the CRISPR array. (B) The natural pathway of antiviral defense involves association of Cas9 with the anti-repeat-repeat RNA (tracrRNA: crRNA) duplexes, RNA co-processing by ribonuclease III, further trimming, R-loop formation, and target DNA cleavage. (C) Details of the natural DNA cleavage with the duplex tracrRNA:crRNA. With the gradual perfection of CRISPR / Cas9 systems, *S. pyogenes* Cas9 (SpCas9) co-expressed with custom guide RNAs (sgRNAs or tracrRNA:crRNA duplexes) have been successfully used in bacteria, fungi, viruses, parasites, plants, animals and human cell lines. Targeting with multiple sgRNAs was also successfully applied to multiplex genome engineering. The 'humanized' CRISPR / Cas9 system has overturned previous methods of animal model generation, functional genomic screens, transcriptional modulation, epigenetic control and live imaging of the cellular genome. However, although CRISPR / Cas9 has a broad range of actions, several aspects still require further investigation and there are some points that remain worthy of attention.

Natural DNA repair mechanisms restore breaks caused by Cas9

DNA is not an inert structure. The genome is susceptible to potentially mutagenic threats of both endogenous and environmental origin. Unless quickly repaired, DNA breaks can lead to chromosomal deletion, loss, rearrangement or cytotoxicity. DNA double-strand breaks (DSBs) are a common form of DNA damage and DSB rejoining is a fundamental mechanism of genome protection. Double-strand break repair mechanisms rely on enzymes that evolved primarily to deal with developmentally programmed double-strand breaks. The deliberate targeted introduction of DNA double-strand breaks by endonucleases and their coordinated repair underpin key biological processes such as recombination between homologous chromosomes during meiosis[52,53].

Nucleases like Cas9-induce DSBs. The principle of genome editing relies on a DNA repair system that works when DNA DSBs occur. In eukaryotic cells, there are two main types of DNA double-strand breaks repair mechanisms, Non-Homologous End-Joining (NHEJ) and homologous recombination (HR) repair. NHEJ rejoins the broken ends and is often accompanied by loss / gain of some nucleotides, thus the outcome of NHEJ is variable: nucleotide insertions, deletions, or nucleotide substitutions in the broken region. HR uses homologous DNA as a template to restore the DSBs, and the outcome of this kind of repair is precise and controllable. For example, through HR repair an exogenous DNA sequence can be added at the break site in the genome.

The strategy of homologous recombination relies on the fact that replication generates an identical copy of the cellular DNA and the undamaged copy can be used as a template for re-synthesis and repair of a damaged DNA strand. Homologous recombination requires extensive stretches of DNA sequence homology but is then a very accurate method of repair. HR is used when generating transgenes that carry extra genetic material By contrast, non- homologous end joining is a much more robust but low fidelity form of double-strand break repair, where the broken ends are simply fused together again irrespective DNA sequence. This process requires at most a few base pairs of homology between the two broken ends, but may lead to loss of nucleotides at the join, hence the alternative names of 'illegitimate recombination' or 'error-prone recombination'. NHEJ is very important when generating mutants with complete loss of a particular gene or protein.

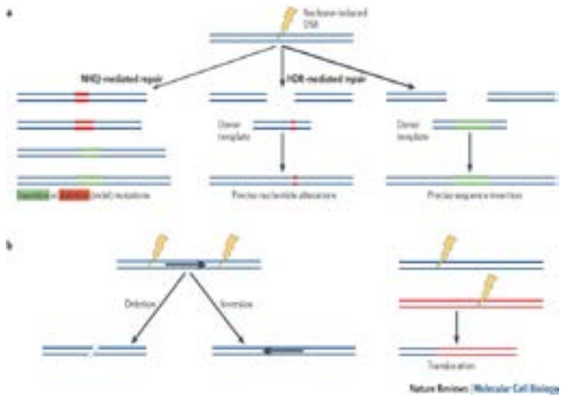

Figure 3: Nuclease-induced DSBs repair mechanisms.

Source:

a) Single nuclease-induced DSBs in a gene locus can be repaired by either NHEJ or HDR. NHEJ-mediated repair leads to the introduction of variable length insertion or deletion (indel) mutations. HDR with double-stranded DNA 'donor templates' can lead to the introduction of precise nucleotide substitutions or insertions. b) The introduction of two nuclease-induced DSBs in cis on the same chromosome can lead to the deletion or inversion of the intervening sequence (left panel). The introduction of two nuclease-induced DSBs on two different chromosomes can lead to the creation of a translocation (right panel).

The experimental set up of CRISPR/Cas for genome engineering mainly composed of four steps.

Select the desired Cas9 cutting site at the gene of interest. Cas9 targets can be any 20-bp DNA sequence followed at the 3′ end by 5′-NGG-3′.

Design and clone specific guide RNA into vector for coexpres-

sion. sgRNA expression vectors can be constructed by cloning 20-bp target sequences into a plasmid backbone encoding a U6 promoter-driven sgRNA expression cassette and Cas9.
Delivery to the cell, this can be done by transfection or electroporation.
Analysis of genomic alteration[54].

Advantage and disadvantage of CRISPR / Cas9 technique

To date, the CRISPR / Cas9 system has already shown itself to comprise a robust and flexible tool for genome editing and gene regulation. Comparing with ZFNs and TALENs, there are several advantages to CRISPR / Cas9. ZFNs and TALENs are built on protein guided DNA cleavage, which needs complex and time-consuming protein engineering, selection and validation. In contrast, CRISPR / Cas9 only needs a short programmable gRNA for DNA targeting, which is relatively cheap and easy to design and produce. Through using Cas9 and several gRNA with different target sites, CRISPR / Cas9 is able to simultaneously induce genomic modifications at multiple independent sites. This technology can accelerate the generation of transgenic animals with multiple gene mutations, and disrupt multiple genes or a whole gene family to investigate gene function.

With further research on CRISPR, however, it became apparent that this technology was not as easy as once assumed. A large number of studies have investigated diverse factors affecting the CRISPR/Cas9 system, such as Cas9 activity, target site selection and sgRNA design, delivery methods, off-target effects, and the incidence of HDR. By addressing these potential pitfalls, we can take better advantage of this technique, as well as improve its efficiency and specificity.

Another disadvantage of the method is because of the adaptability behavior of viruses. Viruses escape immunity by making point mutations in either the memorized regions of their genomes. Mutated virus DNA molecules adopt a stealth mode inside the cell and require a new cycle of memory formation before they are subject to interference once again. Cycles like these contribute to the ongoing coevolution between invaders and their hosts.

Applications of the CRISPR / Cas9 technique in veterinary medicine

One of the most important applications of CRISPR / cas9 system is genome editing. CRISPR / Cas9 provides a robust and multiplexable genome editing tool, enabling researchers to precisely manipulate specific genomic elements, and facilitating the function elucidation of target genes in biology and diseases. It was used in different animals to create gene mutations. also used to induce desired genomic alterations in plants for generating specific traits, such as valuable phenotypes or disease resistance. The production of transgenic animals is now quick and easy through this technique[50,55].

Genetically engineered animal models are crucial for the study of complex cellular and physiological processes. While mouse models have been widely used, the CRISPR / Cas9 gene-editing approach has been established in many other animal models, including worm, fly, fish, rat, rabbit, goat, sheep, dog, pig, and monkey. The expansion of transgenic animal models beyond mouse is advantageous to biomedical research because it can accelerate the development of new therapeutic strategies.

The other possible, yet to be developed application of CRISPR / Cas9 technology is gene therapy. Genome editing has the potential to permanently cure diseases through disrupting endogenous disease-causing genes, correcting disease-causing mutations or inserting new protective genes. As the newest engineered nucleases, CRISPR / Cas9 provides a novel highly efficient genome editing tool for gene therapy studies. For instance, it is possible to remove the integrated proviral viral genes from host cell genomes by CRISPR / Cas9, where Cas9 digests the genes required for viral survival and replication[56,57]. This system could be used for treating infectious diseases by eradicating pathogen genomes from infected individual animal or by mutating the protein used for entry of a particular pathogen. In addition, this system has been shown to delete viral genes from the host cell chromosome.

One of the powerful applications of the CRISPR / Cas9 technology is the high-throughput screening of genomic functions. The oligo libraries encoding hundreds of thousands of sgRNAs can be computationally designed and chemically synthesized to target a broad set of genome sequences. By pairing with Cas9 or dCas9 fusion proteins, this provides an approach to systematically knock out, repress, or activate genes on a large scale. The technique requires a delicate delivery method that ensures that every cell only receives a single sgRNA, usually via lentiviral or retroviral delivery into mammalian cells[58].

In addition to applications in genome editing and regulation, DNA-binding proteins, such as ZFs, TALEs, and dCas9 have been fused to fluorescent proteins (FPs) to allow direct imaging of genomic loci in living cells. Additionally, dCas9 has also been used for studying proteins that interact with specific loci, and it may potentially be used to target RNA[59]. Fluorescently tagged Cas9 labeling of specific DNA loci was recently developed as a powerful live-cell-imaging.

CRISPR / Cas9 gene-editing technology has been used in veterinary research. It is reported that the technology is efficient to generate transgenic animals such as cattle, pigs, chicken, and goats[59-61]. In the realm of livestock, genome editing is just beginning to be applied, so specific applications are still emerging. One example that is being pursued currently is the genetic dehorning of dairy cattle to avoid invasive, painful and expensive physical dehorning procedures[62]. Another application envisioned for cattle and for pigs is a mutation of the myostatin gene, which negatively regulates the production of skeletal muscle[63].

Recently, scientists successfully produce live calves with increased resistance to bovine tuberculosis. They inserted into the natural resistance-associated macrophage protein-1 (NRAMP1) gene into the genome of bovine fetal fibroblasts (a cell derived from female dairy cows). These cells were then used as donor cells in a process called somatic cell nuclear transfer, where the nucleus of a donor cell carrying the new gene is inserted into an egg cell, known as an ovum, from a female cow. Ova were cultivated in the lab into embryos before being transferred into mother cows for a normal pregnancy cycle. The cows later delivered calves harboring the NRAMP1 gene and these calves were resistant to Mycobacterium bovis infection[60].

The other importance of CRISPR / Cas9 gene editing in veterinary medicine is the production of long hair fibers in Cashmere goats. The disruption of fibroblast growth factor 5 (FGF5) in goats results in increased fiber length in cashmere goats and

that these phenotypes have the potential to transmit to the following generations 59. In addition, CRISPR / Cas9 mediated gene-edited pigs that are protected from porcine reproductive and respiratory syndrome virus (PRRS)[64].

For veterinarians there are three specific takeaways:

- CRISPR can be used in animals, specifically companion animals, to remove genetic defects and enhance certain traits.
- Gene editing through CRISPR is a more consumer-friendly technique to get crops and farm animals with greater disease resistance and productivity with enhanced nutritional traits.
- The CRISPR technology can be used in vaccine production, reducing allergenic effects when the vaccines are produced in eggs and potentially speeding up the design of new vaccines[65].

Although CRISPR / Cas9 is still a technology on progress, it will likely help developing countries including Africa to increase livestock productivity and to combat diseases such as those transmitted by vectors. The other promising application of the system is to prolong the aging process of highly productive animals, generate disease-resistant breeds and to make ease the process of vaccine production. At places where some particular disease is endemic and causes a substantial distraction on animals, it will not be a challenge to edit animals' genetic constitution and make the subsequent generation resistant to that particular disease[56].

Technologies like CRISPR / Cas9 could in the future lead to more meaningful inventions that will help combat the spread of deadly diseases, especially in less developed countries. It could be applied to create genetically modified insects. This has a significant importance for the potential control of vector-borne diseases such as trypanosomiasis. Manipulating insect genes to make them more susceptible to insecticides and the production of sterile and harmless insects is a possibility using CRISPR / Cas9.

Perspective of CRISPR/Cas9 regarding Ethical Considerations

The era of straight forward genome editing raises ethical questions that will need to be addressed by scientists and society at large. How can we use this powerful tool in such a way as to ensure maximum benefit while minimizing risks? It will be imperative that nonscientists understand the basics of this technology sufficiently well to facilitate rational public discourse. Regulatory agencies will also need to consider how best to foster responsible use of CRISPR-Cas9 technology without inhibiting appropriate research and development.

An important ethical issue in research is that benefits must be greater than risks. Greater attention must be placed on risks, since they may damage living beings or the environment. The application of the CRISPR / Cas9 technique involves risks since it may produce off-target mutations, which can be deleterious. A high frequency of off-target effects has been found in human cells, but low in mice and zebrafish. One problem is that large genomes may contain multiple DNA sequences identical or highly homologous to the intended target DNA sequence. CRISPR / Cas9 may cleave also these unintended sequences causing mutations that may cause cell death or transformation. Efforts have been made to reduce off-target mutations, but further improvement is needed, especially for precise modifications needed for therapeutic interventions. Another important problem is the efficient safe delivery of CRISPR-Cas9 into cell types or tissues that are hard to transfect and/or infect[66].

The implications of designer animals, consumer-friendly agricultural production and superior vaccination techniques all could be just over the horizon. Undoubtedly much of this brings up ethical questions and questions in regulatory decision-making, but the speed of development of the technology, particularly in countries such as China, is now moving forward so quickly that even with some forms of restrictions and agreements, we will soon see CRISPR cause tectonic shifts in the way the world produces food[65]. The release of CRISPR-edited insects carrying gene drives will require stringent pre-release risk assessment of non-target effects to prevent unintended ecological consequences. In order to assess the technology's potential and avoid the considerable pitfalls, many failures, successes and cautionary tales born of traditional control methods must be considered: they must be precise, must be aware of ecosystem-wide implications, and must be wary of and anticipate possible unintended consequences[67].

Conclusion

Genetic engineering has been extensively used in human and animal science studies. Moreover, it has been used to increase the productivity and environmental adaptability of animals and crops and also to fight against vector-borne communicable diseases. The programmability of the CRISPR / Cas system has made gene targeting and editing much more flexible. With rapid progress in this new technology, there is no doubt that other exciting CRISPR/Cas-related applications would appear in the near future. Moreover, the CRISPR / Cas researches in mammalian cells and animals are providing new insight into clinical applications and promising a new tool for gene therapy. As a powerful, yet versatile, gene-editing and regulation tool, CRISPR / Cas9 technology is already accelerating both research and therapeutics. It is believed that its broad applications in genomics research and cell biology research will greatly advance knowledge of both basic biology and diseases in the years to come. Despite its useful application, optimization of most techniques was laborious, cost-ineffective, non-specific and had many unwanted environmental damages. Since its advent, CRISPR / cas9 is showing a great promise of being the novel genome editing technology of the coming future with a hope to be used in human and veterinary medicine.Besides, this technology used to produce disease-resistant animals especially for exotic breeds that are susceptible for most of endemic diseases.

References

1. Nair, A, J. Introduction to biotechnology and genetic engineering: (2008) Laxmi Publications Ltd.
   Pubmed| Crossref| Others
2. Nicholl, D.S. An introduction to genetic engineering: Cambridge University Press. (2008)
   Pubmed| Crossref| Others
3. Glick, B.R., Pasternak, J.J., Patten, C.L. Molecular biotechnology.

(2010)
Pubmed| Crossref| Others

4. Griffiths, A., Miller, J., Suzuki, D., et al. Making recombinant DNA. An Introduction to Genetic Analysis 7th edition . (2000) WH Freeman.
Pubmed| Crossref| Others
5. Alberts, B., Johnson, A., Lewis, J., et al. Molecular Biology of the Cell 4th edn (New York: Garland Science). (2002) Ann Bot 91: 401.
Pubmed| Crossref| Others
6. Lodish, H., Berk, A., Zipursky, S.L., et al. Molecular cell biology 4th edition. (2000) National Center for Biotechnology Information.
Pubmed| Crossref| Others
7. NHGRI. National Human Genome Research Institute (NHGRI). (2017).
Pubmed| Crossref| Others
8. Brandenberg, O., Dhlamini, Z., Edema, R., et al. Module A-Introduction to molecular biology and genetic engineering. (2011) Food and Agriculture Organization of the United Nations.
Pubmed| Crossref| Others
9. Brookhouser, N., Raman, S., Potts, C., et al. May I cut in? Gene editing approaches in human induced pluripotent stem cells. (2017) Cells 6(1): 5.
Pubmed| Crossref| Others
10. Miller, J. C., Holmes, M.C., Wang, J., et al. An improved zinc-finger nuclease architecture for highly specific genome editing. (2007) Nature biotechno 25(7): 778.
Pubmed| Crossref| Others
11. Doudna, J.A., Charpentier, E. The new frontier of genome engineering with CRISPR-Cas9. (2014) Science 346(6213): 1258096.
Pubmed| Crossref| Others
12. Shi, T-Q., Gao, J., Wang, W.J., et al. CRISPR/Cas9-based genome editing in the filamentous fungus Fusarium fujikuroi and its application in strain engineering for gibberellic acid production. (2019) ACS synthetic biology 8(2): 445-454.
Pubmed| Crossref| Others
13. Ishino, Y., Shinagawa, H., Makino, K., et al. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. (1987) J bacterial 169(12): 5429-5433.
Pubmed| Crossref| Others
14. Lander, E.S. The heroes of CRISPR. (2016) Cell 164(1-2): 18-28.
Pubmed| Crossref| Others
15. Mojica, F.J., Díez-Villaseñor, C., Soria, E., et al. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. (2000) Molecular microbiology 36(1): 244-246.
Pubmed| Crossref| Others
16. Hsu, P.D., Lander, E.S., Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. (2014) Cell 157(6): 1262-1278.
Pubmed| Crossref| Others
17. DNA replication, recombination and repair. Biochemistry 5th edition.
Pubmed| Crossref| Others
18. Nemudryi, A. A., Valetdinova, K. R., Medvedev, S. P., et al. TALEN and CRISPR/Cas genome editing systems: tools of discovery. (2014) Acta Naturae 6(3): 19-40.
Pubmed| Crossref| Others
19. McGarty, T. P. Prostate cancer prognostic tests: Pre and post diagnosis. (2015) Draft white paper.
Pubmed| Crossref| Others
20. Brouns, S.J. A Swiss army knife of immunity. (2012) Science 337(6096): 808-809.
Pubmed| Crossref| Others
21. Horvath, P., Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. (2010) Science 327(5962): 167-170.
Pubmed | Crossref | Others
22. Serrat, X., Esteban, R., Guibourt, N., et al. EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. (2014) Plant methods 10(1): 5.
Pubmed | Crossref | Others
23. Teng, Y., Bennett, M., Evans, K.E., et al. A novel method for the genome-wide high resolution analysis of DNA damage. (2010) Nucleic acids research 39(2): e10.
Pubmed | Crossref | Others
24. Henry, I.M., Nagalakshmi, U., Lieberman, M.C., et al. Efficient genome-wide detection and cataloging of EMS-induced mutations using exome capture and next-generation sequencing. (2014) The Plant Cell 26(4): 1382-1397.
Pubmed | Crossref | Others
25. Li, C.L., Santhanam, B., Webb, A.N., et al. Gene discovery by chemical mutagenesis and whole-genome sequencing in Dictyostelium. (2016) Genome research 26(9): 1268-1276.
Pubmed | Crossref | Others
26. Hoshijima, K., Jurynec, M., Grunwald, D. Precise genome editing by homologous recombination. (2016) Methods Cell Biol 135: 121-147.
Pubmed | Crossref | Others
27. Joung, J.K., Sander, J.D. TALENs: a widely applicable technology for targeted genome editing. (2013) Nat Rev Mol Cell Biol 14(1): 49-55.
Pubmed | Crossref | Others
28. Sander, J.D., Joung, J.K. CRISPR-Cas systems for editing, regulating and targeting genomes. (2014) Nat Biotechnol 32(4): 347-355.
Pubmed | Crossref | Others
29. Erciyes, K. Introduction to Molecular Biology. (2015) Distributed and Sequential Algorithms for Bioinformatics: Springer 11-25.
Pubmed| Crossref| Others
30. Barrangou, R. RNA-mediated programmable DNA cleavage. (2012) Nature biotechnology 30(9): 836.
Pubmed | Crossref | Others
31. Jinek, M., Chylinski, K., Fonfara, I., et al. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. (2012) Science 337(6096): 816-821.
Pubmed| Crossref| Others
32. Bobis-Wozowicz, S., Osiak, A., Rahman, S.H., et al. Targeted genome editing in pluripotent stem cells using zinc-finger nucleases. (2011) Methods 53(4): 339-346.
Pubmed | Crossref | Others
33. Gaj, T., Gersbach, C.A., Barbas, C.F. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. (2013) Trends in biotechnology 31(7): 397-405.
Pubmed | Crossref | Others
34. Carroll, D., Beumer, K.J. Genome engineering with TALENs and ZFNs: repair pathways and donor design. (2014) Methods 69(2): 137-141.
Pubmed | Crossref | Others
35. Boch, J., Bonas, U. Xanthomonas AvrBs3 family-type III effectors: discovery and function. (2010) Annu Rev Phytopathol 48: 419-436.
Pubmed | Crossref | Others
36. Chen, B., Gilbert, L.A., Cimini, B.A., et al. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. (2013) Cell 155(7): 1479-1491.
Pubmed | Crossref | Others
37. Chen, K., Shan, Q., Gao, C. An efficient TALEN mutagenesis system in rice. (2014) Methods 69(1): 2-8.
Pubmed | Crossref | Others
38. Cseke, L.J., Kirakosyan, A., Kaufman, P.B., et al. Handbook of molecular and cellular methods in biology and medicine: CRC press;

2016.
Pubmed | Crossref | Others
39. Harrison, M.M., Jenkins, B.V., O'Connor-Giles, K.M., et al. A CRISPR view of development. (2014) Genes & development 28(17): 1859-1872.
Pubmed | Crossref | Others
40. Kühn, R., Wefers, B. Editing and investigating genomes with TALE and CRISPR/Cas systems: genome engineering across species using TALENs. (2014) Methods 69(1): 1.
Pubmed | Crossref | Others
41. Pellagatti, A., Dolatshad, H., Valletta, S., et al. Boultwood J. Application of CRISPR/Cas9 genome editing to the study and treatment of disease. (2015) Archives of toxicology 89(7): 1023-1034.
Pubmed | Crossref | Others
42. Peng, R., Lin, G., Li, J. Potential pitfalls of CRISPR/Cas9-mediated genome editing. (2016) FEBS J 283(7): 1218-1231.
Pubmed | Crossref | Others
43. Choi, K.R., Lee, S.Y. CRISPR technologies for bacterial systems: current achievements and future directions. (2016) Biotechnol Adv 34(7): 1180-1209.
Pubmed| Crossref | Others
44. Van der Oost, J., Jore, M.M., Westra, E.R., et al. CRISPR-based adaptive and heritable immunity in prokaryotes. (2009) Trends in biochemical sciences 34(8): 401-407.
Pubmed | Crossref | Others
45. Choudoir, M.J., Panke-Buisse, K., Andam, C.P., et al. Genome surfing as driver of microbial genomic diversity. (2017) Trends in microbiology 25(8): 624-636.
Pubmed | Crossref | Others
46. Heidelberg, J.F., Nelson, W.C., Schoenfeld, T., et al. Germ warfare in a microbial mat community: CRISPRs provide insights into the co-evolution of host and viral genomes. (2009) PLoS One 4(1): e4169.
Pubmed | Crossref | Others
47. Agari, Y., Sakamoto, K., Tamakoshi, M., et al. Transcription profile of Thermus thermophilus CRISPR systems after phage infection. (2010) Journal of molecular biology 395(2): 270-281.
Pubmed | Crossref | Others
48. Terns, M.P., Terns, R.M. CRISPR-based adaptive immune systems. (2011) Current opinion in microbiology 14(3): 321-327.
Pubmed | Crossref | Others
49. Barrangou, R., Marraffini, L.A. CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. (2014) Molecular cell 54(2): 234-244.
Pubmed | Crossref | Others
50. Zhang, F., Wen, Y., Guo, X. CRISPR/Cas9 for genome editing: progress, implications and challenges. (2014) Human molecular genetics 23(R1): R40-R46.
Pubmed| Crossref | Others
51. Nguyen, T.H., Anegon, I. Successful correction of hemophilia by CRISPR/Cas9 genome editing in vivo: delivery vector and immune responses are the key to success. (2016) EMBO mol med 8(5): 439-441.
Pubmed| Crossref| Others
52. Barnes, D.E. Non-homologous end joining as a mechanism of DNA repair. Current biology. (2001) 11(12): R455-R457.
Pubmed| Crossref| Others
53. Karran, P. DNA double strand break repair in mammalian cells. (2000) Curr Opin Genet Dev 10(2): 144-150.
Pubmed| Crossref| Others
54. Trevino, A.E., Zhang, F. Genome editing using Cas9 nickases. (2014) Methods enzymol 546: 161-174.
Pubmed| Crossref| Others
55. Wang, F., Qi, L.S. Applications of CRISPR genome engineering in cell biology. (2016) Trends cell boil 26(11): 875-888.
Pubmed| Crossref| Others
56. Zhang, Y., Huang, H., Zhang, B., et al. TALEN-and CRISPR-enhanced DNA homologous recombination for gene editing in zebrafish. (2016) Methods cell boil 135: 107-120.
Pubmed| Crossref| Others
57. Ebina, H., Misawa, N., Kanemura, Y., et al. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. (2013) Sci Rep 3: 2510.
Pubmed| Crossref| Others
58. Shi, J., Wang, E., Milazzo, J.P., et al. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. (2015) Nat Biotechnol 33(6): 661-667.
Pubmed| Crossref| Others
59. Wang, X., Cai, B., Zhou, J., et al. Disruption of FGF5 in cashmere goats using CRISPR/Cas9 results in more secondary hair follicles and longer fibers. (2016) PLoS One 11(10): e0164640.
Pubmed| Crossref| Others
60. Gao, Y., Wu, H., Wang, Y., et al. Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. (2017) Genome boil 18(1): 13.
Pubmed| Crossref| Others
61. Oishi, I., Yoshii, K., Miyahara, D., et al. Targeted mutagenesis in chicken using CRISPR/Cas9 system. (2016) Sci Rep 6: 23980.
Pubmed| Crossref| Others
62. Carlson, D.F., Walton, M.W., Fahrenkrug, S.C., et al. Precision editing of large animal genomes. (2012) Adv Genet 80: 37-97.
Pubmed| Crossref| Others
63. McPherron, A.C., Lee, S.-J. Double muscling in cattle due to mutations in the myostatin gene. (1997) Proc Natl Acad Sci 94(23): 12457-12461.
Pubmed| Crossref| Others
64. Whitworth, K.M., Rowland, R.R., Ewen, C.L., et al. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. (2015) Nature biotechnology 34(1): 20.
Pubmed| Crossref| Others
65. Connolly, J. The politics and crisis management of animal health security. (2016) Routledge
Pubmed| Crossref| Others
66. Rodriguez, E. Ethical issues in genome editing using Crispr/Cas9 system. (2016) j clin Res Bio: 7: 2.
Pubmed| Crossref| Others
67. Taning, C.N.T., Van Eynde, B., Yu, N., et al. CRISPR/Cas9 in insects: Applications, best practices and biosafety concerns. (2017) J insect physiol 98: 245-257.
Pubmed| Crossref| Others