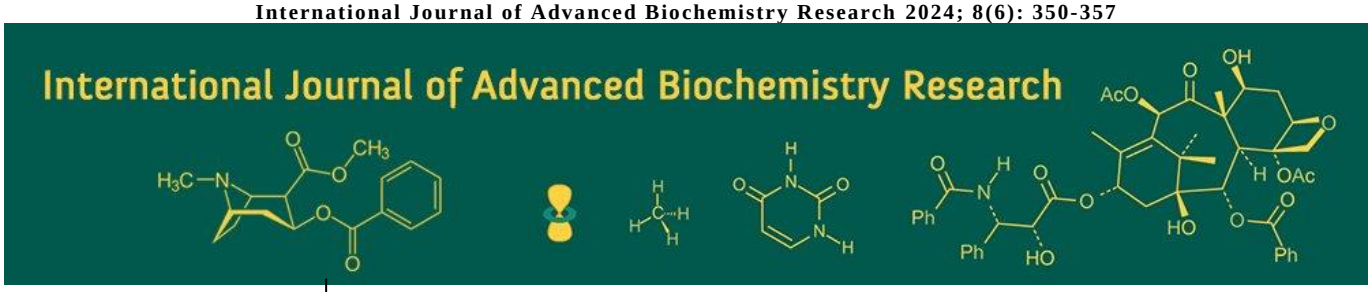

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Navigating the frontier: Crispr's evolution in animal science and the roadblocks ahead

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Abstract
This review delves into the groundbreaking CRISPR/Cas9 technology, showcasing its diverse applications in genome editing, gene therapy, drug discovery, disease modeling, and combatting antibiotic resistance. From addressing genetic disorders and infectious diseases to enhancing food security, CRISPR/Cas9 offers multifaceted solutions across fields like medicine and agriculture. In gene therapy, it shows potential in treating diseases such as sickle cell disease and cystic fibrosis through targeted gene modifications in clinical trials. In drug discovery, CRISPR/Cas9 expedites the identification of therapeutic targets and aids in developing novel treatments by precise genetic modifications in cells and animal models. Additionally, it presents innovative strategies for managing infectious diseases by combating antibiotic resistance and viral infections like HIV. In agriculture, CRISPR/Cas9 enables precise genome editing to enhance traits like disease resistance and yield, fostering improved productivity and sustainability. Despite these challenges, CRISPR/Cas9 emerges as a versatile tool with immense potential in addressing diverse health and agricultural and related issues.

Keywords: CRISPR/Cas-9, genome editing, therapeutic potential, agriculture and livestock, challenges

Introduction
Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems are currently in the spotlight of active research in biology. (Makarova and Koonin., 2015) [52]. Y. Ishino discovered the first CRISPRs in 1987 while studying the gene encoding alkaline phosphatase's isozyme conversion in *Escherichia coli* (Ishino *et al.* 1987) [34]. CRISPR's purpose was unclear until the mid-2000s. Initially spotted in archaea in 1993, they later appeared in more bacterial and archaeal genomes. Similarities with sequences from viruses and plasmids suggested their role in immunity. Concurrently, genes called cas, associated with CRISPR, were identified in hyperthermophilic archaea, further linking CRISPR to defense mechanisms. (Haft *et al.*, 2005; Makarova *et al.*, 2006) [28, 53]. Analogous to the eukaryotic RNA interference (RNAi) system, comparative genomic analysis therefore showed that CRISPR and Cas proteins (the cas gene products) actually cooperate and form an acquired immunity system to protect prokaryotic cells against invasive viruses and plasmids (Brouns *et al.*, 2008; Hale *et al.*, 2009) [11, 30]. The first human use of CRISPR-Cas in 2016 marked a milestone following its initial demonstration in 2012 by George Church, Jennifer Doudna, Emmanuelle Charpentier, and Feng Zhang, with Doudna and Charpentier later awarded the 2020 Nobel Prize for their work. The pivotal 2007 experiment in Streptococcus thermophilus showcased CRISPR-Cas's role in acquired immunity by conferring resistance to phage attacks and limiting plasmid transformation. Yoshizumi Ishino and colleagues discovered CRISPRs in *E. coli* in 1987, while metagenomic analysis by Andersson and Banfield revealed dynamic changes in CRISPR loci sequences. Subsequent research demonstrated the heterologous protection provided by the CRISPR-Cas system of *S. thermophilus* against plasmid transformation and phage infection, highlighting Cas9's essential role in CRISPR-encoded interference. Genome editing using the CRISPR-Cas system of *Streptococcus pyogenes* further solidified its recognition as a prokaryotic acquired immunity system. This review focuses on type II-C CRISPR systems' biology, mechanism, and applications, primarily Cas9s (Chen *et al.*, 2023) [15].

Structural and biochemical studies

Cas9 possesses a bilobed structure, with the guide RNA nestled between the nuclease and alpha-helical lobes. A solitary bridge helix connects these two lobes. The RuvC nuclease domain cleaves the non-target DNA strand, whereas the HNH nuclease domain cleaves the target DNA strand, both found in the multi-domain nuclease lobe. Sequentially dissimilar sites that interact in the tertiary structure to generate the RuvC cleavage domain encode the RuvC domain (Jinek *et al.*, 2012; Doudna *et al.*, 2014; Jinek *et al.*, 2014). Target DNA must have a protospacer adjacent motif (PAM), made up of the three-nucleotide sequence NGG, which is an essential characteristic. The PAM-interacting domain (PI domain), situated close to Cas9's C-terminal end, is responsible for identifying this PAM. Cas9 transitions between the apo, guide RNA-bound, and guide RNA: DNA-bound states through discrete conformational changes. Cas9 can determine the CRISPR locus's intrinsic stem-loop topology, which is responsible for forming the crRNA-tracrRNA ribonucleoprotein complex (Sternberg *et al.*, 2014; Jiang *et al.*, 2015; Jinek *et al.*, 2014). Cas9 further recognizes and breaks down the target dsDNA when it forms a complex with trans-activating crRNA (tracrRNA) and CRISPR RNA (crRNA). A chimeric single-guide RNA that has been shown to have the same function as the original RNA complex takes the place of the crRNA-tracrRNA complex.Cas9 anchors the sgRNA base in a T-shaped architecture coupled with the target ssDNA (Jinek *et al.*, 2012; Jiang *et al.*, 2017; Nishimasu *et al.*, 2014). The DNA-bound Cas9 enzyme's crystal structure reveals the position of the HNH domain in addition to unique conformational changes in the alpha-helical lobe about the nuclease lobe. The protein consists of a recognition lobe (REC) and a nuclease lobe (Jiang *et al.*, 2017; Nishimasu *et al.*, 2014; Anders *et al.*, 2014)

Mechanisms of crispr/cas-9 genome editing

CRISPR/Cas-9 genome editing involves three main steps: recognition, cleavage, and repair. (Jinek *et al.*, 2012; Doudna *et al.*, 2014; Jinek *et al.*, 2014) [37, 23, 39]. The engineered sgRNA guides Cas-9 to the target gene, triggering a double-strand break (DSB) three base pairs upstream of the PAM sequence. Cas-9 then initiates local DNA melting, forming an RNA-DNA hybrid, followed by cleavage of the DNA strands. Repair occurs through non-homologous end joining (NHEJ) or homology-directed repair (HDR), with NHEJ being error-prone and HDR requiring a homologous DNA template for precise gene editing (Sternberg *et al.*, 2014; Jiang *et al.*, 2015; Jinek *et al.*, 2014) [39].

Delivery of crispr-cas systems

Various methods exist for introducing CRISPR-Cas systems into cells, primarily differing in the delivery of the gRNA and Cas9 protein. The gRNA can be supplied as RNA or DNA cloned into a plasmid, while the Cas9 protein can be delivered as a protein, transcribed mRNA, or plasmid DNA (Yin *et al.*, 2019; Staahl *et al.*, 2017; Zuris *et al.*, 2015) [97, 75, 102]. Delivery of the Cas9 protein as a ribo-protein complex offers advantages due to its transient presence and minimal off-target effects (Zuris *et al.*, 2015; Ramakrishna *et al.*, 2014; Staahl *et al.*, 2017) [102, 68, 75]. Physical techniques like electroporation, microinjection, and mechanical cell deformation, as well as carriers such as viral vectors (e.g., lentiviruses, adenoviruses, adeno-associated viruses) and non-viral methods (e.g., lipid nanoparticles, polymer nanoparticles, DNA nanostructures), can be used to transfer gRNA and Cas9 into cells (Doudna & Charpentier, 2014; Ramakrishna *et al.*, 2014; Wang *et al.*, 2019) [23, 68, 85]. Both viral and non-viral carriers protect gRNA and Cas proteins, enhancing delivery reliability and efficacy (Wang *et al.*, 2016) [87].

Application of CRISPR-Cas9

Role in Gene Therapy

Gene therapy, a leading advancement in medical biotechnology, involves modifying defective genes and replacing them with healthy DNA. CRISPR/Cas-9, alongside other techniques, has revolutionized gene therapy, with 22 treatments authorized between 1998 and 2019. Notably, CRISPR/Cas-9 holds promise for treating a range of genetic disorders, including sickle cell disease, cystic fibrosis, and Duchenne muscular dystrophy. In sickle cell disease, CRISPR/Cas-9 boosts fetal hemoglobin production by inhibiting the BCL11A gene (Brendel *et al.*, 2016; Frangoul *et al.*, 2021; Traxler *et al.*, 2016; Canver *et al.*, 2015) [9, 24, 81, 14]. Similarly, it shows potential for correcting the CFTR gene mutation in cystic fibrosis (Schwank *et al.*, 2013; McCarron *et al.*, 2020; Alton *et al.*, 2015; Davies *et al.*, 2018) [73, 56, 3, 20] and restoring dystrophin expression in Duchenne muscular dystrophy (Ousterout *et al.*, 2015; Nelson *et al.*, 2016; Tabebordbar *et al.*, 2016; Min *et al.*, 2019) [65, 62, 77, 58]. CRISPR-based therapies have also entered human trials for lung cancer treatment, demonstrating promising results in modifying T-cells to target cancer cells effectively (Nelson *et al.*, 2016; Tabebordbar *et al.*, 2016; Young *et al.*, 2016) [62, 77, 99].

Pipeline of CRISPR-Cas-assisted drug discovery

CRISPR-Cas tools have revolutionized genetic manipulation across various organisms, from human ESCs to the malaria parasite, previously deemed nearly impossible. These advancements accelerate functional genomics, aiding in the discovery and validation of therapeutic targets, particularly in mammalian models and human cells (Akcakaya *et al.*, 2018; Joung *et al.*, 2017) [1, 40]. They offer potential in improving animal models, enhancing safety testing, and refining patient treatment plans. Moreover, CRISPR-Cas editing enables the development of personalized cellular therapies, such as cancer-targeting T cells and reprogrammed induced pluripotent stem cells (iPSCs), for both genetic and non-genetic disorders (Cai *et al.*, 2020; Kosicki *et al.*, 2018; Gaudelli *et al.*, 2017) [13, 45, 25]. In drug development, CRISPR-Cas systems streamline discovery, validation, and safety testing processes without significant delivery or administrative hurdles. This paves the way for novel therapeutic approaches and paradigms, making CRISPR-Cas indispensable in advancing medicine (Tycko *et al.*, 2016; Liang *et al.*, 2015; Doudna & Charpentier, 2014) [82, 50, 23].

CRISPR-based assays for rapid detection of SARS-CoV-2

The COVID-19 pandemic escalated the urgency for widespread testing to detect and mitigate transmission. While qRT-PCR tests are the gold standard, they have limitations. Nucleic acid-based tests offer higher sensitivity, and efforts to enhance efficiency include integrating LAMP-

based isothermal detection. Combining CRISPR-based techniques with isothermal technologies, as proposed by Broughton *et al*. (2020) [10], Joung *et al*. (2020) [41], Lalli *et al*. (2020) [46], promises rapid and sensitive detection of SARS-CoV-2 nucleic acids.

Potential of CRISPR/Cas9 Gene Editing as a Treatment Strategy for Neurological disorders

The distinguishing feature of neurodegenerative illnesses, which include Huntington's disease (HD), Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD), is age-dependent and selective neurodegeneration. These neurodegenerative diseases are more common as human life expectancy increases; the pathophysiology of most of these illnesses is still unknown, and there are currently no reliable treatments for these significant brain malfunctions (Ascherio & Schwarzschild, 2016; Brown & Al-Chalabi, 2017; Bang *et al.*, 2015) [5, 12, 6]. Despite decades of understanding Alzheimer's disease (AD) at the molecular level, finding effective treatments has been challenging. Clinical trials targeting beta-amyloid have often fallen short, prompting a need to explore alternative therapies (Cummings *et al.*, 2021; Selkoe & Hardy, 2016; Herrup, 2015; Schneider *et al.*, 2014) [17, 34, 31, 72]. Cas9-mediated knock-in mutations can aid in creating animal models for diseases caused by mutant protein toxicity, such as Huntington's disease. (Dabrowska *et al.*, 2018; Monteys *et al.*, 2017; Xu *et al.*, 2017) [18, 59, 94].

CRISPR/Cas9 in cancer immunotherapy

Current treatment medications often have high toxicity and low success rates, despite efforts in immunotherapy and chemotherapy to control cancer cell growth. Additionally, evolving mutations in proto-oncogenes and tumor-suppressive genes limit the effectiveness of multi-targeted therapies to a few carcinogenesis pathways. (Barata *et al.*, 2021; Vasan *et al.*, 2019; Mehta *et al.*, 2020) [7, 84, 57]. CRISPR/Cas9 corrects causal mutations with minimal toxicity and enhances immunotherapy. However, key drawbacks include ethical concerns, generic limitations upstream of the Protospacer Adjacent Motifs (PAM) leading to off-targ*et al*terations, and a lack of consensus on risk assessment. (Pickar-Oliver & Verma *et al.*, 2020) [67]. Animal models are crucial in refining CRISPR/Cas9 experiments for improved genome editing specificity and enhanced anti-tumor responses. Clinical trials utilize the CRISPR/Cas9 system in immune cells for precise genome modifications. Recent advancements in error-free *in vitro* technologies aim to overcome limitations of this gene-editing system. The article focuses on using CRISPR Cas9 technology to treat treatment-resistant cancers. (Manguso *et al.*, 2017; Marceau *et al.*, 2016; Morselli *et al.*, 2015) [54, 55, 60]. Additionally, the use of CRISPR/Cas9 is aided as an emerging supplementation of immunotherapy, currently used in experimental oncology.

CRISPR and HIV: New technique in human blood unveils potential paths toward cure

CRISPR/Cas-9 gene-editing technology shows promise in treating microbially-induced infectious diseases, particularly HIV/AIDS. Temple University researchers demonstrated in animal models that deleting the HIV-1 genome using CRISPR/Cas-9 halted replication and removed the virus from infected cells (Yin *et al.*, 2017; Wang *et al.*, 2016; Kaminski *et al.*, 2016; Dash *et al.*, 2019) [96, 87, 42]. CRISPR/Cas-9 can also modify genes encoding the chemokine coreceptor type-5 (CCR5) in host cells, blocking HIV entry (Xu *et al.*, 2017; Wang & Cannon, 2016; Liang *et al.*, 2016; DiGiusto *et al.*, 2016) [94, 85, 49, 21]. T cells from human blood were genetically modified using CRISPR-Cas9, deleting multiple genes. When exposed to HIV, cells lacking essential viral replication genes showed reduced infection, while those lacking antiviral components showed increased infection (Wang *et al.*, 2015; Zhang *et al.*, 2021; D'Orso, 2017) [91, 101].

CRISPR-Cas system: A potential alternative tool to cope antibiotic resistance

Antimicrobial resistance (AMR) poses a global health threat, fueled by the transfer of AMR genes among bacterial pathogens via horizontal gene transfer (HGT). To combat this, new approaches are urgently needed (Tacconelli *et al.*, 2018; Homes *et al.*, 2016;). CRISPR/Cas systems, originally a prokaryotic immune mechanism, have emerged as powerful tools for combating AMR by targeting and cleaving DNA sequences encoding antibiotic resistance genes (Bikard *et al.*, 2014; Citorik *et al.*, 2014) [98, 8]. Given the slow pace of antibiotic development compared to bacterial evolution, alternative strategies such as bacteriophage therapies, antibacterial peptides, bacteriocins, and anti-virulence chemicals are crucial in tackling antibiotic-resistant infections (Imai *et al.*, 2019; Tacconelli *et al.*, 2018) [33, 79-80]. The CRISPR-Cas system has been leveraged for molecular recording and selectively targeting antibiotic resistance genes, offering promise in combating AMR (Yosef *et al.*, 2015; Bikard *et al.*, 2014) [98, 8].

CRISPR/Cas9 technology on cardiac research: From disease modelling to therapeutic approaches

Genome-editing technology, particularly the CRISPR/Cas9 system, has been widely used to correct DNA mutations, ranging from single base pairs to large deletions, in both *in vitro* and *In vivo* models, enhancing our understanding of cardiovascular disorders like lipid metabolism and electrophysiology (Savarese & Lund, 2017; Wu, 2017; Kessler *et al.*, 2015) [71, 93, 43]. CRISPR/Cas9 facilitates gene knockout or knockin in human cells, particularly in induced pluripotent stem cells (iPSCs), offering valuable insights into disease mechanisms (Savarese & Lund, 2017; Wu, 2017; Musunuru, 2013; Kessler *et al.*, 2015) [71, 93, 43]. Despite its potential, challenges related to biology, technology, and ethics hinder its therapeutic application in cardiovascular diseases (Savarese & Lund, 2017; Wu, 2017; Kessler *et al.*, 2015) [71, 93, 43]. Remarkably, CRISPR/Cas9 has demonstrated efficacy in correcting genetic defects in postnatal/adult mice, exemplified by its ability to edit the *PCSK*9 gene, resulting in reduced blood cholesterol levels and lowered risk of coronary heart disease (CHD) (Wang *et al.*, 2017; Ran *et al.*, 2015) [90, 91].

CRISPR in livestock and poultry: From editing to printing

Since the advent of the CRISPR revolution, precise genome editing of large animals has been achievable for application in biomedicine and cattle. The process is not necessarily a simple, quick, or safe path from editing to printing, or from genetic engineering to producing the required animals. Selecting the optimum method for genome editing, embryo

generation, zygote microinjection or electroporation, cryopreservation, and embryo transfer is necessary when using CRISPR in large mammals. These procedures can be time-consuming and expensive (Hai *et al.*, 2014; Whitworth *et al.*, 2014) [29, 92]. The primary technological advancements and frequently asked questions to enhance this revolutionary biotechnology in big animals. In light of the rising worldwide demand for food, CRISPR improves livestock production in several ways, including efficiency gains, a decrease in the environmental effect of farming, improved pest management, and improved animal welfare and health. It is no longer a technical challenge. For the first time in the CRISPR era, debates and agreements, chances and dangers, advantages and disadvantages, ethics and science should all be reexamined (Lassner & Peterson, 2015; Larson *et al.*, 2013) [48, 47]. Over time, CRISPR technology has evolved and improved, enabling it to create transgenic bird lines primarily for food purposes, especially for producing meat or eggs. The application of CRISPR technology may result in the sustainable and effective development of poultry products, so assisting in addressing issues related to global food security (Oishi *et al.*, 2016; Van *et al.*, 2020) [64, 83]. Growth, feed conversion, digestibility, increased egg output, and general improved performance of chickens raised for meat and eggs could all be significantly impacted by CRISPR technology. Technological developments in CRISPR may also improve disease resistance, vaccination delivery, and immune response. This will lead to improvements in the health of the poultry, the safety of immunizations derived from chicken eggs, and the production and safety of food (Lillico *et al.*, 2013; Guo *et al*,. 2021) [51, 26]. Gene inactivation by indels introduction is known as knockout (KO). Efficiency of the CRISPR/Cas9 tool ranges from 10% to over 90% in a variety of animals and cell types, including human, sheep, goat, cattle, pig, and mouse. Prior to the commercial release of CRISPR/Cas9 ribonucleoprotein (RNP), KO efficiency was dependent on plasmid transfection. However, RNP delivery offers a greater KO efficiency and circumvents the drawbacks of using DNA plasmid delivery (Zhang *et al.*, 2021; Wang *et al.*, 2021) [101, 88]. After CRISPR/Cas9 RNP delivery, the RNP activates quickly to perform DSB, and indels are visible very quickly. Since RNP is removed from the cells in less than a day, there is a lower chance of off-target mutations. Plasmid delivery, on the other hand, carries the danger of inadvertent off-target mutation and may even contribute to a vector's integration into the host genome (Kim *et al.*, 2014; Richardson *et al.*, 2016) [44, 70].

Anti-crispr proteins

Anti-CRISPR proteins, found in phages, hinder the normal function of the bacterial immune system CRISPR-Cas, effectively reversing its unintended effects. They act as blockers of Cas proteins, allowing phages to evade CRISPR-mediated defense mechanisms. Before the discovery of anti-CRISPR proteins, phages relied on acquiring mutations to reduce their binding affinity to CRISPR, but bacteria could counter this through "priming adaptation." Anti-CRISPR proteins are now recognized as the most effective strategy for ensuring phage survival during bacterial infections (Pawluk *et al.*, 2018; Altae-Tran *et al.*, 2023) [66, 2].

Ethical issues

CRISPR-Cas9 technology offers affordable, precise genome editing with applications spanning human health, agriculture, and environmental conservation. However, ethical concerns, especially regarding germline editing in humans and environmental impacts, must be addressed through global legislation. While regulations are necessary, they shouldn't hinder scientific progress. This article explores CRISPR-Cas9's uses and ethical dilemmas across various domains, emphasizing the need for comprehensive ethical deliberation.

Conclusion

CRISPR is becoming an indispensable tool in biological research. Once known as the CRISPR is rapidly developing into a vital tool for biological study. The programmable ability of the Cas9 enzyme, once recognized as the bacterial immune system against invasive viruses, is transforming an array of industries, including biotechnology, medical research, and the livestock industry. These days, CRISPR-Cas9 is used for more than only editing genes. Catalytically hindered inactive Cas9 has applications in chromatin engineering, imaging, gene regulation, and epigenetic editing. By learning about and comprehending these difficulties, we will be better equipped to assess the extent of their constraints and develop strategies for overcoming them. Undoubtedly, CRISPR-based technologies will continue to revolutionize biotechnological, clinical, and basic research. There are some challenges in store, though. The possible immunogenicity of CRISPR-Cas9 proteins is one such barrier. Therefore, further study must be done to deal with the safety and specificity of such technologies in tandem with the existing improvements. In addition, enough thought must be given to the ethical and social ramifications of these technologies for them to be useful to all societal strata and to benefit all of animal and humankind.

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