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Application of CRISPR-Cas9 mediated genome editing in different fungi of veterinary importance

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Abstract
Opportunistic mycoses cause a wide range of diseases in animals and birds, from localized infections to fatal disseminated diseases, such as aspergillosis, candidiasis, cryptococcosis, and infections especially caused by mycotoxins. The manipulation of fungal genomes is critical for understanding the function of target genes, pathobiology of fungal infections, virulence potential, and pathogenicity of clinically important fungi, as well as the development of new diagnostics and therapeutic targets. In this review, we provide a thorough introduction to the CRISPR/Cas9 system and a summary of the most recent fungal genome editing applications using CRISPR/Cas9. This technology has been successful in elucidating the pathogenesis of fungal species and the interactions between hosts and pathogens. It has also produced novel insights into the susceptibility to and resistance to antifungal drugs, as well as creative treatment plans and an increase in the secondary metabolite content of fungi.

Keywords: CRISPR, Cas9, mycoses, gene editing, *Candida*, homologous recombination

1. Introduction
The fungi represent a large, diverse group of eukaryotic microorganisms comprising both yeasts and molds that have a variable genome size, and short time frames for growth and reproduction (Lin *et al*., 2015) [24]. Invasive fungal infections have emerged as an important cause of animal mortality, particularly for avians/birds. *Aspergillus*, *Candida*, *Blastomyces*, *Histoplasma* and *Cryptococcus* are the major fungal genera that cause opportunistic and life-threatening mycoses in both humans and animals (Schmiedel *et al*., 2016) [36]. *Histoplasma* has a global distribution and causes pulmonary and systemic infections in a wide range of mammals, including humans and especially *Histoplasma capsulatum var. farciminosum* affects the skin and the subcutaneous lymphatic system in equids (horses, donkeys and mules) called epizootic lymphangitis (Guillot *et al*., 2018) [16]. In animals, aspergillosis is primarily a respiratory infection. *Aspergillus* causes Aspergillosis in poultry and severely affects the lower respiratory system (Seyedmousavi *et al*., 2018) [38]. while in ruminants, it causes mycotic pneumonia, gastroenteritis, mastitis, placentitis and abortions. *Aspergillus* species also cause guttural pouch infections, keratomycosis and pneumonia in horses (Dobesova *et al*., 2012) [9] and bronchopulmonary infections in dogs and cats (Sharman *et al*., 2012) [40]. The aflatoxins produced by *Aspergillus* are the most potent natural carcinogen and hepatotoxic compounds (Da Rocha *et al*., 2014) [6]. The dimorphic *Blastomyces* causes a very serious disease in dogs called as the canine blastomycosis which is characterized by Ocular involvement and ulcerative cutaneous lesions (Schwartz, 2018) [37]. *Cryptococcus* are encapsulated fungal pathogens and the etiological agents of *Cryptococcosis*, that infects animals causing mastitis and pneumonia in bovines, meningitis in equines and very serious rhinitis in cats referred to as *Feline cryptococcosis* worldwide (Refai *et al*., 2017) [35]. Dermatophytosis is caused by dermatophytes and is characterized by superficial invasion of the hyphae in the skin, hair, and nails causing subacute or chronic infections (Burstein *et al*., 2020) [2]. Candidiasis is the most common mycotic disease caused by *Candida albicans*, which are ubiquitous ascomycetes yeast that normally inhabit the alimentary and genital mucosa, mouth, oesophagus and skin of animals and humans. *Candida* severely affects the mucosa of crop in chickens, turkeys and other avian species, causing thrush, crop mycosis and sour crop (Dhama *et al*., 2013) [7].

All these examples show the importance of fungi in animal disease production related to skin, digestive or respiratory system.

These fungi have been subjected to in-depth molecular genetic analysis in order to identify factors involved in their virulence, interactions with the host, resistance to antifungal agents and other critical biological processes (Dudakova *et al*., 2017) [10]. Genome editing technology is a versatile engineering tool for the genetic manipulation of microorganisms such as fungi (Gaj *et al*., 2016) [14]. Clustered regularly interspaced short palindromic repeat (CRISPR)-based technology is one example of a genetic tool that has revolutionized targeted genetic manipulation in a variety of fungal and other microbial species (Uthayakumar *et al*., 2021) [44]. The CRISPR/Cas9 genome editing system was first introduced into *Saccharomyces cerevisiae* (DiCarlo *et al*., 2013) [8]. Then subsequently, scientists applied the CRISPR/Cas9 genome editing system to *Trichoderma reesei* (Liu *et al*., 2015) [25], afterward this system was applied to the model fungi *Neurospora crassa* and *A. nidulans* (Matsu-Ura *et al*., 2015) [28]. respectively. Since then, the CRISPR/Cas9 genome editing system has found wide applications in the genetic alteration of many fungi. CRISPR-based technologies have recently been used to perform targeted genetic mutations and deletions in *Candida albicans* and other *Candida* species (Nargesi *et al*., 2021) [31].

2. Mechanism of Action of CRISPR-Cas9

The CRISPR/CRISPR-associated protein 9 (Cas9) is an adaptive immune system in bacteria and is a prokaryote-derived genome editing system that can perform sequence deletions, insertions, substitutions, integrations and epigenetic genes regulation (Newsom *et al*., 2021) [32]. In 1987, researchers found the CRISPR systems for the first time in *E. coli* (Ishino *et al*., 1987) [18]. The CRISPR/Cas system can be divided into two categories, with six major types (Shmakov *et al*., 2017) [41]. The type II CRISPR/Cas9 system is currently widely used in various species as a simpler CRISPR system. The type II system consists of nuclease (Cas9), mature CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) and RNaseIII. Additionally, a single guide RNA (sgRNA) complex can be created by the combination of the crRNA and tracrRNA (Jinek *et al*., 2012) [19]. The trans-activating crRNA (tracrRNA) and the CRISPR RNA (crRNA), which are two separate RNAs, respectively, activate and direct the Cas proteins in a specific manner (Yang *et al*., 2021) [51].

The Cas9 protein, which is functionally an endonuclease pairs with the single guide RNA (sgRNA), forming a sgRNA-Cas9 complex that comprises of a Cas9-binding region (the conserved sgRNA "tail") and a unique 20 nucleotide "N20" region complementary to the targeted genomic locus. This sgRNA-Cas9 complex interacts with the target locus based on the complementary binding of the sgRNA N20 to the target region, assuming that the target locus also contains the required protospacer adjacent motif (PAM) (Wensing *et al*., 2019) [49]. The target sequence must be adjacent to a protospacer adjacent motif (PAM) in the genome for the CRISPR complex to target the correct genomic location. The short PAM sequence allows the CRISPR complex to differentiate between the target and the sgRNA sequence (La Russa *et al*., 2015) [23]. The PAM sequence of SpCas9 from S. pyogenes that is currently used in *Aspergillus* is 50-NGG-30 (Hsu *et al*., 2013) [17]. After binding, the Cas9 endonuclease undergoes a conformational change, resulting in a double-stranded break (DSB) within the DNA region (Palermo *et al*., 2017) [34]. This DSB can then be repaired using either nonhomologous end joining (NHEJ) or homology-directed repair (HDR). The HDR mechanism allows for precise gene modification through homologous recombination between the donor template and the repair DNA whereas, NHEJ can cause genomic changes by inducing random base loss, insertion, and replacement at DSB positions. For CRISPR-based genetic modification in *Candida* and other yeast species, the HDR mechanism has been most frequently used (Vyas *et al*., 2018) [46].

3. Applications

Here are some applications of the CRISPR-Cas9 genome editing tool in clinically important fungi.

3.1 Genetic modification of *Candida* species using CRISPR

In 2015, Vyas *et al*. used CRISPR for the first time in C. *albicans*. They modified a Cas9 nuclease and sgRNA system adopted from S. cerevisiae and made it work best in C. *albicans*. They composed two systems, a solo and a duet system, wherein one or two plasmids were utilized to express Cas9 and the sgRNA, which were both directed towards targeting the ADE2 gene. In this technique, a single transformation was capable of producing homozygous gene knockouts and numerous gene mutations in both alleles of the diploid *Candida*. The systems displayed 20-40% and 60-80% mutation frequencies for the duet and solo systems, respectively (Vyas *et al*., 2015) [45].

Min *et al*. used a transient system that expressed Cas9 and sgRNA which mainly targeted the ADE2 gene of *Candida albicans*. The DSB repair method was used to ablate the ADE2 gene and a repair template containing a marker for nourseothricin resistance selection was provided. The transient system successively targeted numerous loci by incorporating multiple sgRNA cassettes into a single cell (Min *et al*., 2016) [29].

Other approaches, such as the C. *albicans* LEUpOUT system and the HIS-FLP system, have been developed to enable high-efficiency, markerless homozygous CRISPR editing, as well as marker recycling and CRISPR removal (Nguyen *et al*., 2017) [33]. Nguyen and colleagues recently presented a fast and efficient edition that used permanent markers in the engineering location rather than a molecular cloning phase.

Shapiro *et al*., (2018) [39] developed a C. *albicans* double-gene deletion library as well as a CRISPR-Cas9-based gene drive platform in 2018. In this case, HDR was used to insert into the NEUT5L locus a plasmid containing CAS9, two sgRNAs rather than one, which was flanked by areas homologous to the target gene. When this mutant strain was crossed with a wild strain of the opposite mating type, a homozygous double-gene deletion mutant was produced because the cell still contains the machinery required to disrupt the second wild-type allele.

3.2 Deciphering *Candida* host-pathogen interactions using CRISPR techniques

C. albicans is recognized by the host when components of the fungal cell wall interact with the pattern recognition receptors of innate immune cells. The interaction between innate receptor Dectin-1 with the β-glucans of the cell wall of *C. albicans* is essential to mount an immune response, however,

C. albicans has evolved strategies to mask β-glucans and avoid detection. By using the CRISPR-Cas9 system mutants were generated that lacked protein kinase A (PKA), iron homeostasis regulators (Ftr1 and Sef1) and phosphatidylserine synthase (Cho1). The absence of these genes disrupted the signaling pathways involved in β-glucan masking and activated Several Mitogen Activated Protein Kinase (MAPK) pathways like Cek1 which resulted in the unmasking of β-glucan (Chen *et al*., 2019) [3].

C. albicans can survive within the macrophage phagosome by utilizing alternative carbon sources for its metabolism and promote fungal survival, reduced macrophage survival, increased phagosome acidity, and increased hyphal formation. Single-, double-, triple-, and quadruple-mutant strains lacking the genes which are involved in amino acid, dicarboxylic acid, and N-acetylglucosamine metabolism were generated using CRISPR. This increased macrophage survival and decreased hyphal growth inside macrophages (Williams *et al*., 2020) [50].

Similarly, the disruption of the MNN4-like gene family, of C. *albicans* was found to increase the immune response and promote the ability of macrophages to phagocytose fungal cells by positively regulating phosphomannan expression on the cell wall (González-Hernández *et al*., 2017) [15].

3.3 The use of CRISPR-Cas9 to transform *Cryptococcus neoformans*

Cryptococcosis is a life-threatening fungal disease caused by Cryptococcus neoformans and *Cryptococcus gattii* which are found in soils with bird droppings, and in association with rotting vegetation, including eucalyptus tree hollows (Elhariri *et al*., 2016) [11]. The genome of *Cryptococcus neoformans* was edited using a transient CRISPR-Cas9 expression (TRACE) system. The capsule of *Cryptococcus* is the major virulence factor and is encoded by the CAP64 gene. The disruption of this gene led to the loss of both capsule and virulence, thus hampering its pathogenicity (Zhang *et al*., 2021) [52].

3.4 CRISPR used to study antifungal drug susceptibility and resistance

The mechanism of action of most of antifungal drugs targets components of the fungal cell wall. Antifungal drugs that are used for therapy against *Candida* infections such as azoles and polyenes targets ergosterol, while drugs such as echinocandins target the enzyme (1,3)-β-D-glucan synthase. CRISPR-Cas9 technology has been utilized to decipher the intricate mechanisms of resistance. Studies have confirmed that valine substitution is sufficient to impart resistance.

According to a study conducted by Kannan *et al*., 2019 [20], a CRISPR-Cas9 gene-targeting cassette was used to introduce single base-pair mutation via HDR, in which due to valine to glycine V668G substitution in the transcription factor MRR1 resulted in resistance to both fluconazole, and the pyrimidine 5-fluorocytosine (5-FC) which was accompanied by upregulation of the multidrug transporter MFS7 (Kannan *et al*., 2019) [20].

Manogepix (MGX), is another antifungal drug, that prevents proper cell wall synthesis by targeting glycosylphosphatidylinositol (GPI) biosynthesis enzyme Gwt1. According to a study by Kapoor *et al*., 2019 [21], performed gene editing by CRISPR, to create the valine to alanine V163A substitution in the Gwt1 protein in *C. glabrata*, which resulted in decreased susceptibility to MGX (Kapoor *et al*., 2019) [21].

Antifungal resistance is also conferred by efflux pumps and by targeting two antifungal efflux pumps at the same time could help overcome drug resistance. CRISPR–Cas9-based 'gene drive array' created the homozygous double-deletion mutants platform which, targeted the antifungal efflux pump. By deleting the two-efflux pump genes, TPO3 and YOR1, the susceptibility towards a drug was also increased as a result of which *C. albicans* became highly sensitive to fluconazole and other antifungal drugs (Shapiro *et al*., 2018) [39].

The ribonucleoprotein (RNP) based CRISPR/Cas9 approach in *A. fumigatus* is used to correct mutations in the genes which are responsible for antifungal drug resistance like azoles such as itraconazole, voriconazole, posaconazole and isavuconazole. The sterol-demethylase-encoding gene *cyp51A* and 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase-encoding gene, *hmg1* were corrected in the majority of triazole-resistant *Aspergillus fumigatus.* Also, the protein kinases encoding genes *sepL* and *sidB* were disrupted by the RNP-based CRISPR/Cas9 system, which hampered the Septation Initiation Network (SIN). This led to a decline in viability in response to the antifungal drug such as echinocandin stress, accompanied by loss of hyphal septation with increased susceptibility to cell wall stress, as well as widespread damage of hyphae and loss of virulence, characterized by lack of tissue invasive growth (Souza *et al*., 2021) [42].

3.5 CRISPR/Cas9 fitness analysis of the dimorphic fungal pathogen *Blastomyces dermatitidis*

Blastomyces dermatitidis is a dimorphic fungal pathogen affecting the lungs. CRISPR/Cas9 for high-frequency gene targeting and editing in Blastomyces revealed that the secreted zincophore PRA1 and its transporter ZRT1 are necessary for disease development. Blastomyces have two genes, PRA1 and ZRT1, that are involved in zinc scavenging and uptake from the extracellular environment. Under zinc-limiting conditions, the disruption of PRA1 or ZRT1 by CRISPR/Cas9 had a varied effect on growth, showing reduced growth at early time points and lowering the fitness of the fungus post infection (Kujoth *et al*., 2018) [22]. The ability of C. *albicans* to infiltrate epithelial cells is also hampered by gene ablation (Citiulo *et al*., 2012) [4].

3.6 Detection of zoonotic dermatophytes by Cas12a

The development of a CRISPR-based diagnostic platform provides the possibility of a rapid, and accurate diagnostic tool that is otherwise less accurate and time consuming than the conventional diagnostic methods such as Wood's lamp examination, microscopic identification and fungal culture. Dermatophytosis is an infectious disease of hair, nails, and skin caused by several fungal species such as *Microsporum canis, Nannizzia gypsea and Trichophyton mentagrophytes*, which are zoonotic in nature. Wang *et al*., 2021 [47] developed a Cas12a-based assay in conjunction with recombinase polymerase amplification (RPA) to distinguish these three major zoonotic dermatophytes. The RPA-Cas12a method shows high sensitivity and specificity (100% and 100%, respectively) (Wang *et al*., 2021) [47].

3.7 CRISPR/Cas9-assisted secondary metabolites regeneration in filamentous fungi

As the most prevalent airborne fungal pathogenic species found in nature, *A. fumigatus* is becoming an increasingly lethal threat to immunocompromised animals. *Aspergillus fumigatus* produces Trypacidin, which is a natural antimicrobial antibiotic compound and potent toxin to lung cells, and causes phagocytosis of cells such as alveolar macrophages. Through genome editing, the functional re-establishment of the gene cluster for trypacidin biosynthesis was accomplished by the elimination of a single adenosine insertion at the 3881 position of polyketide synthase (PKS) which encodes the gene tynC in a non-producing strain. As a result, the insertion of adenosine in the genome was deleted using CRISPR/Cas9 to restore trypacidin (Weber *et al*., 2017) [48]. Mycotoxin elimination in *Aspergillus* can similarly be carried out by CRISPR by following the study conducted by Liu *et al*., 2015 [25]. In this study Monascus purpureus produces a mycotoxin, called as citrinin which is nephrotoxic and deletion of the 15-kb citrinin biosynthetic gene cluster was performed using a dual-plasmid CRISPR/Cas system referred to as a large genomic fragment (LGF) deletion. As a result, citrinin production was hampered and an increase in pigmentation was witnessed by 2 to 5% (Liu *et al*., 2020) [26].

3.8 CRISPR-based detection of complement proteins

Histoplasma capsulatum (Hc), a fungal pathogen, invades, replicates and destroys macrophages. C3aR signaling is essential for the rapid capture of fungi by macrophages, according to genome-scale CRISPR screening. We conducted a host-directed CRISPR-Cas9 screen to investigate the molecular mechanisms underlying this interaction and identified genes that alter macrophage susceptibility to Hc infection (Cohen *et al*., 2022) [5].

Table 1: Applications of the CRISPR/Cas system in important fungi like the C. *albicans* and non-*Candida albicans*, *Aspergillus*, *Rhizopus* and *Mucor*

Organism	Target Gene (S)	Purpose of Application	References
C. albicans	ADE2, CDR1/CDR2	Duet and Solo systems are used to generate homozygous mutations in a single transformation.	Vyas *et al*. (2015) [45]
C. albicans	ADE2	A transient CRISPR-Cas9 system for efficient gene deletion is described.	Min *et al*. (2016) [29]
C. albicans	antifungal efflux and biofilm adhesion factors	To create a gene drive array system for the generation of combinatorial deletion mutants.	Shapiro *et al*. (2018) [39]
C. albicans	ADE2, URA3, WOR1, WOR and CZF1	To create a marker-free system that does not require molecular cloning.	Nguyen *et al*. (2017) [33]
C. parapsilosis, C. orthopsilosis, C. metapsilosis and C. tropicalis	ADE2 and CPAR2_101060	To create an autonomously replicating plasmid for markerless editing.	Lombardi *et al*. (2017) [27]
C. glabrata	ADE2, VPK1 and YPS11	To create a loss-of-function mutation using the NHEJ repair pathway	Enkler *et al*. (2016) [12]
A. fumigatus	PKSP	To examine the CRISPR-CAS9 technique in this organism	Fuller *et al*. (2015) [13]
A. fumigatus	pksP and cnaA genes	To develop a system for mutagenesis	Zhang *et al*. (2021) [52]
A. fumigatus	CYP51A	To look at how cyp51A change affects azole resistance mechanisms.	Umeyama *et al*. (2018) [43]
Rhizopus delemar	PYRF	Point mutation was introduced for analysing molecular pathogenic processes	Bruni *et al*. (2019) [1]
Mucor circinelloides	CARB and HMGR2	A plasmid-free CRISPR-Cas9 method was used to produce mitotically stable mutants.	Nagy *et al*. (2017) [30]

4. Conclusions and Future prospects

The CRISPR/Cas9 system is a potent genome editing tool that has been applied to a wide range of industrially important and pathogenic fungi. CRISPR-based genome editing can range from a simple single gene mutation to a complex multi-gene expression regulation, depending on the efficiency of homologous recombination and fungal intrinsic identity. However, various constraints and hurdles must be overcome to develop effective CRISPR/Cas9-mediated genome editing strategies for fungal species. Off-target effects caused by Cas9's non-targeted nuclease activity are a significant barrier to genome editing in fungi using CRISPR technology. Current diagnostic methods for fungal infections are time-consuming and costly, involving phenotypic and biochemical approaches. Thus, there is a greater demand for modern molecular diagnostic methods such as genomic imaging with Cas9. The advancement of CRISPR/Cas9-based genome editing technology would greatly simplify genetic manipulation, advance the study of functional genes, and enhance the generation of recombinant proteins and other natural products in a variety of fungal species.

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