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Al-Laaeiby et al., *Afr. J. Clin. Exper. Microbiol.* 2019; 20 (4): 260-267 https://www.afrjcem.org

African Journal of Clinical and Experimental Microbiology ISSN 1595-689X Oct 2019 Vol.20 No.4
AJCEM/1943: https://www.ajol.info/index.php/ajcem

Copyright AJCEM 2019. https://dx.doi.org/10.4314/ajcem.v20i4.1

**Review Article** **Open Access**

# *Candida* species: the silent enemy

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## Abstract:

*Candida* species are known to cause serious infections in immunocompromised patients but uncommon cases have been reported in immunocompetent individuals regardless of the harmless co-existence of the fungi with the host. Recently, the incidence rate of candidiasis has increased dramatically alongside the emergence of antifungal resistance. Although conventional methods to ensure prompt diagnosis of candidiasis for effective therapy have been established, the scientific world is witnessing progress in the development of more accurate, timely and cost-effective methods that is coinciding with the molecular revolution and advanced DNA analysis. Moreover, the challenges of resistance of *Candida* to available antifungal agents are being met with the deployment of molecular techniques to investigate the mechanisms of resistance. This review is an attempt to provide up-to-date information on the persistent problems of *Candida* with highlights on the clinical importance, molecular diagnosis, and resistance to candidate antifungal drugs; azoles and echinocandins.

**Keywords:** *Candida*, resistance, molecular diagnosis, azole, echinocandin

Received April 1, 2019; Revised May 13, 2019; Accepted May 30, 2019

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# Espèce *Candida*: l'ennemi silencieux

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## Abstrait:

Les espèces de *Candida* sont connues pour causer des infections graves chez les patients immunodéprimés, mais des cas peu communs ont été rapportés chez des individus immunocompétents, indépendamment de la coexistence inoffensive des champignons avec l'hôte. Récemment, le taux d’incidence de la candidose a considérablement augmenté parallèlement à l’émergence d’une résistance antifongique. Bien que les méthodes conventionnelles permettant d’assurer un diagnostic rapide de la candidose en vue d’un traitement efficace aient été établies, le monde scientifique constate des progrès dans la mise au point de méthodes plus précises, plus rapides et plus rentables qui coïncident avec la révolution moléculaire et l’analyse avancée de l’ADN. De plus, les défis posés par la résistance de *Candida* aux agents antifongiques disponibles sont résolus par le déploiement de techniques moléculaires pour étudier les mécanismes de résistance. Cette revue tente de fournir des informations à jour sur les problèmes persistants de *Candida* en soulignant l’importance clinique, le diagnostic moléculaire et la résistance aux antifongiques candidats; les azoles et les echinocandins.

**Mots-clés:** *Candida*, résistance, diagnostic moléculaire, azole, echinocandine

## Introduction:

The scientific term *Candida* was derived from the Latin name ‘candid’, referring to its white colour (1). Anton van Leeuwenhoek first observed yeast cells under the microscope in 1680 (2) and classified it into kingdom Fungi, phylum Ascomycota, class Saccharomycetes, order Saccharo-mycetales, family Saccharomycetaceae and

genus *Candida* (3). The significant feature of yeast is the ability to alter its morphology from unicellular ovoid cells to pseudohyphae and hyphae forms. They are therefore known as dimorphic fungi (4). The morphological transition of yeast is stimulated by surrounding conditions such as temperature, pH, blood, serum and nutrient abundance (4). They form chlamydospores in specific conditions (5) and reproduce non-sexually by forming buds or sexually through mating (4).

*Candida* dwells in human body as a commensal where they are regarded as normal flora. They inhabit different body sites including the skin, mouth, gastro-intestinal tract and vagina, and are generally harmless in immunocompetent individuals, scarcely infecting healthy people with only mild infections (6). However, they become pathogenic opportunistic fungi in individuals with impaired immune system (7).

## Methodology:

During the period, September 2018 to March 2019, we employed Google scholar engine to search for relevant published materials and bibliographic citations including original and review articles, books, and conference papers through the period 1985 to 2018. Keywords employed for the search were Candidiasis, *Candida* diagnosis, *Candida* classification, *Candida* resistance, *Candida* molecular diagnosis, and *Candida* treatment. One hundred and ninety four reference materials were identified but following assessment of the relevance of the materials, only 70 reference materials were selected for the review.

## Clinical significance of *Candida*

*Candida* species of clinical importance include *Candida albicans* and non-albican *Candida* species such as *Candida parapsilosis*, *Candida krusei*, *Candida glabrata*, *Candida dubliniensis, Candida tropicalis, Candida stellatoidea, Candida auris, Candida guilliermondii, Candida lusitaniae* and *Candida kyfer* (8, 9). Clinically, *C. albicans* is of utmost significance because of its frequent isolation as a pathogenic and virulent species compared to other species (10). Genetically, *C. albicans* is diploid with heterozygous cells compared to *C. glabrata* that is haploid (10, 11) . *C. albicans* is also an imperfect yeast (vegetative) as its life cycle contains a non-sexual phase only (10).

*Candida* species can cause infections ranging from simple superficial to life threatening systemic diseases (12). Superficial infections affect the skin, nails and hair in addition to mucous layer (13) and include such diseases as athlete's foot, ring worm, oral and vaginal thrush (14,15). Studies have shown that approximately 75% of females suffer from vaginal *Candida* infections (vulvovaginal candidiasis) at least once during their life (16). *Candida* has been reported as the third or fourth cause of nosocomial infections in the United States (17) and as an insidious pathogen causing ocular candidiasis, cerebral candidiasis or candida meningitis in infants and susceptible adults (8).

Shetti and co-workers reported for the first time in 2011 that *Candida* treatment failure is an indicator of HIV infection (18). Similar to other pathogenic agents, opportunistic pathogenic *Candida* species possess virulence factors that enable colonization and host infection. The stages of infection involve inoculation, adherence to the host tissue, penetration and dissemination to other body sites through the bloodstream (6).

## Risk factors for *Candida* infection

*Candida* infections are associated with multiple risk factors and the prevalence of *Candida* infection is related to the ability of the pathogen to adapt to environmental factors, for example, pathogenic species successfully grow and multiply at 37°C compared to non-pathogenic species (8,13). The risk factors can be categorized into two; (i) factors associated with medical management and (ii) factors related to host immune status.

Recent advances in health care management and medications have provided the opportunity of a suitable environment that supports *Candida* growth and colonisation of the host. Such factors include catheterization, parentheral nutrition, organ transplantation, prolonged hospitalization, and use of new medications (8). On the other hand, host factors such as age, gender, and immune status determine the incidence of candidiasis (8).

An *invitro* study showed that nicotine concentrations of cigarette smoke are associated with yeast growth and adherence in both biofilm and plankton forms (19). Others have shown an association between *Candida* infections and iron deficiency, owing to the relationship of iron and the strength of the immune system (20). Newborns are at risk of infection with *Candida* species because of the risk of transmission from the mother during childbirth and perinatal period (21). Moreover, patients with AIDS, oral cancer, cystic fibrosis, dentures, and recipients of lung, liver, stem cell or other solid organs are

highly susceptible to *Candida* infections (6, 22)

## Laboratory diagnosis of *Candidiasis*

The accurate diagnosis of infection caused by pathogenic microorganisms is an important guide in the selection of appropriate treatment regimen (23). The diagnosis of fungi infection is complex due to overlapping symptoms and signs with other infections, patients' age, health status and environmental factors (24). Advances in the field of mycology have led to the development of diagnostic methods for fungi infections (1). Two mycological laboratory approaches are employed in the diagnosis of *Candida* infections; conventional standard (microscopy and culture) and non-culture methods such as serology and molecular techniques (25) (Fig 1).

In the era of molecular diagnosis, new techniques have been developed and used for accurate identification of fungi (26). However, the conventional methods are still employed in combination with the new advanced molecular techniques (27). As explained by Kurtzman *et al*., the conventional methods are necessary for the understanding of the fungi and their ecological interactions in addition to obtaining accurate results through molecular analysis of their gene variations and modifications (28).

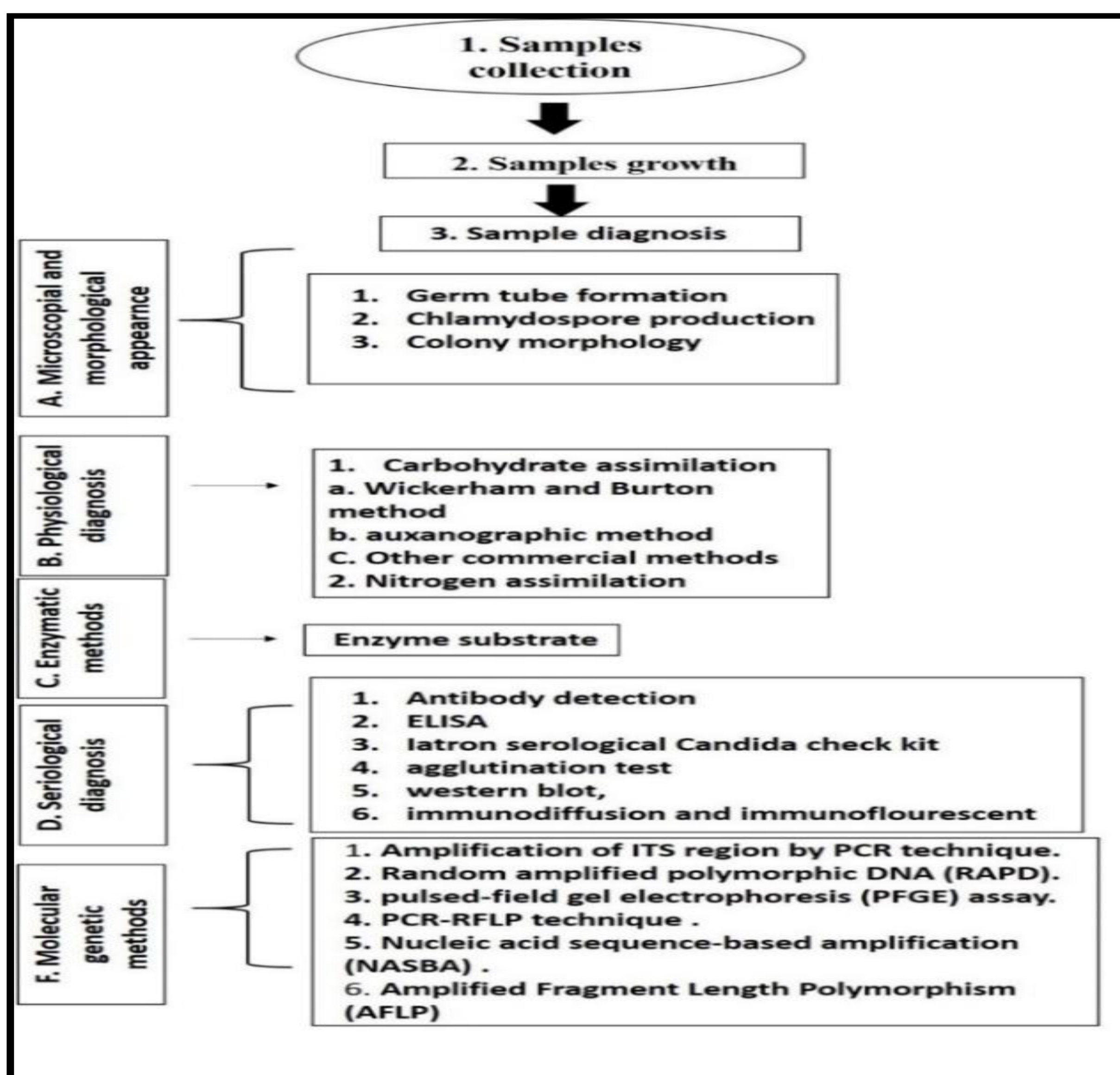


Figure 1: Schematic diagram summarizing important steps for laboratory diagnosis of *Candida*

## Molecular identification of *Candida*

***Polymerase chain reaction (PCR) assay***

PCR technique is useful for basic molecular identification of the organism, however successful PCR depends on DNA extraction, purity and quantity (29). The amplification by PCR involves three phases of denaturation, annealing (hybridization) and extension. To amplify specific target DNA sequence in a conventional PCR, specific primers are designed to hybridize to the sequence of target and amplification carried out in master mix PCR reaction tubes inside a thermal cycler machine. PCR products are then separated on an agarose gel to visualize the DNA bands and confirm successful DNA fragment amplification (29).

PCR techniques are promising assays that evaluate and differentiate yeast and pathogenic fungi species. In particular, the multiplex PCR is a simple, timely and cost effective approach to identify different species of yeasts (30). Luo *et al*., skipped the DNA extraction step and amplified DNA directly from the yeast colony. This modification reduced the turn-around-time for the assay and avoided the obstacle of DNA isolation which reduced the overall cost (30).

The conserved sequences in the genome of organisms exhibit divergence among different species, the knowledge of these variations have been utilized to determine relatedness between species (26). Kurtzman and Robnett investigated the relatedness between yeast species using the variations of D1/D2 domain in a large subunit (26S) of ribosomal DNA, while at the same time, amplifying and sequencing the Internal Transcribed Spacer (ITS) region with universal primers, and comparing this with the BLAST database (31,32).

There are two ITS regions, which are non-coding regions located between the small and large subunits of rRNA. In fungi, ITS1 and ITS2 are located in the rDNA gene complex between the 18s and 5.8s rRNA and 5.8s and 26s rRNA genes respectively (32). These regions are distinguished by their variations among fungal species and have therefore been selected as DNA barcode to differentiate the fungal species (33). Leaw *et al*., have demonstrated the candidate ITS regions for *Candida* species identification. First, the length of the amplified ITS fragments differs among *Candida* species (34). However, the maximum length (size) of the DNA fragment amplified is less than 0.3 kb, with the exception of *Saccharomyces cerevisiae* with length of 3.7 kb and *C. glabrata* with 0.4 kb (34). Secondly, the identification of yeast species, depending on the ITS regions (particularly ITS2) is reliable, accurate and timely (it can be completed within 24 hours) when used in identification of clinical isolates (34). Romeo *et al*. (35) used specific primers targeting the *HWP1* gene to differentiate between *C. albicans*, *C. africana* and *C. dubliniensis* by amplifying different sizes of DNA segments with sizes of 941 bp, 700 bp and 569 bp respectively (35).

***Random amplified polymorphic DNA***

The amplification of random fragments of genomic DNA to investigate genetic diversity among species is called random amplified polymorphic DNA (RAPD) and it has been used to study the relatedness of *Candida* isolates (36). The advantages of the RAPD assay is that of simplicity with no need for precise DNA sequence data, rapidity and lower cost. However, the problem of reproducibility is a major disadvantage because non-specific or arbitrary primers annealed to the DNA under demanding conditions (37,38). The study by Valério *et al* reported that RAPD may be unreliable due to the phenotypic switching in *Candida* species (39).

***Pulsed-field gel electrophoresis assay***

The investigation of the karyotypes of *Candida* species is an additional means of differentiating between *Candida* species which can be achieved with pulsed-field gel electrophoresis (PFGE) assay (38). However, this analytical assay is time consuming and labour intensive.

***Nucleic acid sequence based amplification***

Nucleic acid sequence based amplification (NASBA) is an alternative method to PCR technique that is used to amplify specific segments of rRNA using RNA polymerase to detect active pathogens (40).

***Restriction fragment length polymorphism***

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique has contributed to the precise diagnosis of *Candida* species. Mousavi *et al.,* identified five *Candida* species; *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. guilliermondii* by PCR-RFLP technique (41). The technique involves amplification of DNA segments using universal primers ITS1 and ITS4, followed by digestion of the amplified products using *Msp* I restriction enzyme (41). The digested products are separated on agarose gel and the characteristic band patterns are interpreted according to established protocol.

Table 1: Summary of the targets and actions of antifungal agents (44)

| Antifungal | Target | Action |
|---|---|---|
| Amphotericin B (polyene) | Cell membrane (ergosterol) | Targets the integrity of the cell membrane by binding to ergosterol, owing to its affinity to ergosterol more than any other sterol. The drug affects permeability by forming pores in the cell membrane. |
| Azole | Cell membrane (ergosterol) | Targets the synthesis of ergosterol by inhibiting lanosterol 14-alpha-demethylase, which converts lanosterol to ergosterol in the fungal cell membrane. |
| Echinocandin | Cell wall (glucan) | Targets the integrity of the fungal cell wall by inhibiting B-glucan synthase, leading to the inhibition of glucan synthesis. |
| Flucytosine | DNA and RNA synthesis | 5-Flucytosin uptake is undertaken by the cytosine permease and this interferes with the pathways used for DNA and RNA synthesis. |

## *Candida* treatment and resistance

Four major classes of anti-fungal agents are approved by the United States Food and Drug Administration (FDA); amphotericin, azole, ecchinocandin and flucytosine (42). The mechanism of action of each class is summarised in Table 1. Compared to the wide range of antibacterial drugs, only a few antifungal drugs are available with noticeable side effects due to the similarity of fungal and human cell membrane (both are eukaryotic cells) which is the site of action of a large number of antifungal agents (43). Echinocandins and azoles are the most effective antifungal agents against candidiasis. Echinocandins tend to have fewer side effects than other antifungal classes due to the absence of a cell wall (its target of action) in the human cell (43).

In spite of the success of these classes of anti-fungals for treatment of yeast and mould infections, these fungal pathogens have developed resistance to these drugs (44). Resistance to anti-fungal agents occur through mutational changes in the genome of the fungi leading to modifications of the metabolic pathway of the drugs in the pathogen (44). There are two categories of resistance; primary (intrinsic) and secondary (acquired). Primary resistance is genetically inherited from the parent as seen in human pathogenic fungi such as *Lomentospora prolificans,* which is intrinsically resistant to all antifungal drugs (45). Secondary resistance is acquired from surrounding environment (46).

## Molecular mechanisms of drug resistance in *Candida*

In more recent years, the determination of genes and their product functions have been carried out via molecular and genetic engineering techniques, such as through the determination of the gene mutations which cause clinical failure through gene sequencing or tracking gene expression using real time PCR or microarrays (47,48). *Candida* species have developed various mechanisms to resist antifungal medications depending on the type of drugs, mechanisms of action and drug targets (44). The most commonly available antifungal drugs for treatment of candidiasis are azoles and echinocandins but *Candida* species have developed resistance to them (49).

***Resistance to echinocandins***

Echinocandin inhibits the enzyme, β-1,3-D-glucan synthase, leading to arrest in the biosynthesis of β-1,3-D glucan and disruption of the integrity of the fungal cell wall (43) (Table1). The enzyme is composed of two subunits; catalytic 'Fks' subunit and regulatory 'Rho' subunit though echinocandin targets the *Fks1* gene (43).

*Candida* species have developed resistance to echinocandin despite its effectiveness. The exposure of *C. albicans* to echinocandin induces the synthesis of chitin (50). Two signalling pathways; PKC $Ca^{2+}$-calcineurin and HOG, regulate the expression of the genes for chitin synthesis (51). Both signalling pathways are involved in the activation of transcriptional stimulation in response to echinocandins (50).

Additionally, presence of mutations in the *Fks1* gene is associated with resistance to echinocandin. For example, sequencing of resistant *C. auris* isolates showed substitution of serine to phenylalanine in the *Fks1* gene locus (9). Mutations in the conserved region of the *Fks1* gene are called 'hot spots' for acquisition of *Candida* resistance to ecchinocandin (52).

***Resistance to azoles***

Azole targets ergosterol biosynthesis (Table 1) but *Candida* has developed four mechanisms to circumvent the fungicidal mechanism of azole including an efflux pump to lower drug concentration within the cell, modification of the ergosterol target, elevating levels of production of the targeted enzyme, and the establishment of alternative pathways (44). Some strains showed cross

resistance to antifungal drugs. *C. tropicalis* carried more than one mutations leading to azole-polyene cross resistance (53).

Two types of transporter proteins, which export different elements such as carbohydrate include the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) (54). These proteins are encoded by the *CDR* and *MDR* genes respectively. The efflux pump encoding genes; *FLR1*, *AZR1* and *TPO1* are associated with resistance to flucytosine, ketoconazole and caspofungin respectively (55,56,57). Studies have shown that *Candida* utilised these genes to expel drugs taken by the cell outside of the cells thereby reducing the concentration within the cell to ineffective level (54). The upregulation of transporter genes is thus associated with azole resistance in *C. albicans* (58).

The transcriptional profiling of resistant *C. glabrata* isolates to azole has been compared with their counterpart susceptible isolates using DNA microarray technique. The results showed that the upregulation of *PDR1* genes (related to a single mutation in a different locus of Cg*PDR1* ORF) is associated with azole resistance (59). Northern blot analysis revealed that *CDR1*, *CDR2* and *MDR1* genes were over-expressed in the resistance isolates compared to susceptible strains (60). The disruption of single or double genes involved in the efflux pump generated mutants sensitive to fluconazole rather than the resistance parent strains in the plankton (61).

Another mechanism in which the *Candida* species develop resistance to azole antifungal drugs is by modifying the drug targets (62). *ERG11* gene encodes lanosterol, 14-α-demethylase (Erg11p), which is targeted by the azole antifungal drug (62). The analysis of PCR products (products mediated by gene amplification from sensitive and resistance strains) was used to investigate point mutations of the *CYP51* gene in the resistance isolates that reduced the function of azole (63). Point mutation leads to amino acid substitution leading to decreased binding of the azole to its target. Flowers *et al.,* characterised 26 novel point mutations while Morio *et al.,* highlighted more than 140 mutations leading to amino acid substitutions. Mutations in the genome sequence, involving *ERG3* and *ERG11* genes of *C. tropicalis,* led to development of resistance to azoles (53).

The action of azole against the fungal cells interferes with the synthesis of functional ergosterol and produces a toxic intermediate compound called 14α methyl-3.6-diol, blocking fungi growth (44). However, *Candida* species have developed another mechanism to resist azole drugs by establishing an alternative pathway. For instance, a missense mutation in the *ERG3* gene of *C. tropicalis* leads to accumulation of the sterol intermediates, ergosta-7,22-dienol and ergosta-7-enol instead of ergosterol (64). *C. albicans* strains produce functional sterol 14α-methyl-fecosterol instead of ergosterol, which leads to development of resistance to fluconazole (65). Another study reported that reduction of *C. krusei* susceptibility to azole drugs was related to up-regulation of Erg11p expression (66). The overexpression of the *ERG11* gene is associated with the mutation of the *UPC2* gene (zinc cluster transcription factor) (68). In a study that targeted the *UPC2* gene disruption, there was reduction in *ERG11* expression in the UPC2 deficient mutant compared to wild type strains (67). It was shown that the mutation in the *UPC2* gene coinciding with the upregulation of *Erg11* expression was one of the mechanisms of increase, causing *ERG11* gene over-expression. However, other mechanisms do exist (69).

## Conclusion:

*Candida* species cause a wide range of infections from mild to severe life threatening diseases. Meanwhile, they inhabit the human body as normal flora. It could therefore be said that these normal flora are silent enemies because they exploit opportunities and attack the body in the event of a perturbation of the immune system. Apart from this, *Candida* species have the ability to modify their genome to develop resistance to antifungal drugs. Although, azoles and ecchinocandins are the most common antifungal agents effective against *Candida* infections, resistance to these drugs are increasing.

In recent time, attempts have been made to develop methods of identifying *Candida* species by exploring certain variations in the conserved region of their genome to determine relatedness between species. To solve the problem of increasing resistance to antifungal agents, molecular techniques are being utilized to investigate gene modifications that overcome the action of antifungal drugs. These efforts aspire to develop more accurate, effective and less time-consuming methods of diagnosis in addition to developing new antifungal agents.

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