OPEN ACCESS Journal of Animal Health and Production

**Review Article**

# Molecular Typing and Susceptibility Profile of *Cryptococcus neoformans* and *Cryptococcus gattii* species Complex: An updated Review

**MOHAMMED TAHA[1], YASMINE HASANINE TARTOR[1*], SARA ZAKI IBRAHIM[2], RANA MOHAMED ABD EL-AZIZ[3]**

*[1]Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University, 44511-Zagazig, Sharkia, Egypt; [2]Veterinarian, Faculty of Veterinary Medicine, Zagazig University; [3]Cairo International Airport Veterinary Quarantine, General Organization for Veterinary Services, Ministry of Agriculture, Cairo, Egypt*

**Abstract** | Cryptococcosis is a sub-acute or chronic fungal infection of human and animals that is induced by opportunistic capsulated basidiomycetous yeasts of genus *Cryptococcus*, primarily *C. neoformans* and *C. gattii* with a worldwide distribution. The genetic variability of *C. neoformans/C. gattii* species complex has been investigated by several molecular techniques mainly hybridization and nested and multiplex PCR assays. Other PCR-based methods were employed for identification as PCR fingerprinting, PCR Restriction fragment length polymorphism (PCR-RFLP), Amplified fragment length polymorphism (AFLP), and multi-locus sequence typing (MLST). Moreover, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been recently introduced for *Cryptococcus* identification and subtyping. *Cryptococcus neoformans/C. gattii* species complex is recently divided into seven species: *C. neoformans*, *C, deneoformans*, *C. gattii*, *C. deuterogattii*, *C. bacillisporus*, *C. tetragattii*, and *C. decagattii* in addition to four interspecies hybrids with differences in pathogenicity, epidemiology, and antifungal susceptibility. Long-term usage of antifungal drugs led to the emergence of resistance in *C. neoformans* and *C. gattii* species. Thus, antifungal susceptibility is of great importance in the epidemiological investigation for tracking the susceptibility profiles and drug resistance. Moreover, efficient antifungal therapies selection for cryptococcosis treatment is based on minimum inhibitory concentration (MIC) values of the gold standard drugs for cryptococcosis therapy: amphotericin B, fluconazole, 5-flucytosine, voriconazole, ketoconazole, and itraconazole. This review article highlights the main molecular methods used for identification and genotyping of *C. neoformans* and *C. gattii* and presents the global prevalence and antifungal susceptibility profiles of these environmental isolates against the commonly used antifungals.

**Keywords** | *Cryptococcus neoformans*, *Cryptococcus gattii*, PCR, Molecular types, MALDI-TOF MS, antifungal susceptibility

**Editor** | Asghar Ali Kamboh, Sindh Agriculture University, Tandojam, Pakistan.
**Received** | November 02, 2020; **Accepted** | November 25, 2020; **Published** | December 27, 2020
***Correspondence** | Yasmine Hasanine Tartor, Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University 44511-Zagazig, Sharkia, Egypt; **Email:** jasmen21@yahoo.com; yasminehtartor@zu.edu.eg
**Citation** | Taha M, Tartor YH, Ibrahim SZ, Abd El-Aziz RM (2020). Molecular Typing and Susceptibility Profile of *Cryptococcus neoformans* and *Cryptococcus gattii* species Complex: An updated Review. J. Anim. Health Prod. 9(s1): 17-26.
**DOI** | http://dx.doi.org/10.17582/journal.jahp/2020/9.s1.17.26
**ISSN** | 2308-2801

## INTRODUCTION

*Cryptococcus neoformans/C. gattii* species complex comprises capsulated basidiomycetous yeasts, the primary causative agent of cryptococcosis, which is a worldwide distributing life-threatening fungal disease producing tissue infections, pneumonia, and commonly meningoencephalitis mainly in immunosuppressed patients (Kwon-Chung et al., 2014).

Biological, epidemiological, clinical, pathogenicity, and drug susceptibility differences were observed between the species complex. Biochemically, *C. neoformans* is divided into two varieties (*C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*), while the capsule polysaccharides difference subdivided it into three serotypes: A, D, and AD in addition to serotypes B and C for *C. gattii* (Silva and de Albuquerque Maranhão, 2015). Recently, after various molecular studies, the complex C. *neoformans*/*C. gattii* was rearranged into 7 haploid species: *C. neoformans* var. *grubii*, C. de*neoformans* var. *neoformans*, along with *C. gattii*, C. *bacillisporus*, *C. deuterogattii*, *C. tetragattii*, and *C. decagattii* as well as four interspecies hybrids (Hagen et al., 2015).

*Cryptococcus neoformans* and *C. gattii* have a different and overlapping ecological niche. *C. neoformans* is frequently isolated from birds dropping, especially in pigeon nests, while *C. gattii*, occurring principally in tropical or subtropical areas, is found frequently in soil debris and decaying trees like oaks, pink showers, and Eucalyptus trees (Alves et al., 2016). Almost all of the cryptococcal infections are relevant to the species *C. neoformans* var. *grubii*, particularly in immunocompromised patients and also apparently healthy hosts (Bandalizadeh et al., 2020). One million cryptococcal meningitis cases are reported annually in patients with human immunodeficiency virus infection and acquired immune deficiency syndrome with a mortality peak at approximately 625,000 deaths (Harris et al., 2011). Meanwhile, *C. gattii* causes a smaller fraction of cases affecting commonly immunocompetent individuals in temperate regions (Firacative et al., 2012).

Cryptococcus infection is also very important in a wide range of animals worldwide. It may affect various mammals like cats, dogs, horses, cattle, sheep, goats and birds, with two basic forms: pulmonary and cerebral cryptococcosis. The cutaneous, ocular, osseous, and visceral form may be found due to disseminated infection. Cryptococcosis is mostly associated with mastitis in cattle, sheep, and goats besides endometritis and placentitis in mares (Refai et al., 2017).

Concerning *Cryptococcus* complex epidemiological spreading, *C. neoformans* var. *grubii* (VNI, II, and VNB) is more predominant and distributed globally than *C. neoformans* var. *neoformans* (VNIV), which is observed in Europe (Cogliati, 2013). The VGI and VGII are the most widely distributed among *C. gattii* members and they are responsible mainly for the Vancouver outbreak, which is still ongoing in Canada and USA resulting in more than 350 human cryptococcosis cases, 3.5% of them were lethal even with powerful antifungal therapy. Meanwhile, VGIV is the least distributing genotype associated with cryptococcosis infection in central Africa (Byrnes and Marr, 2011; D'Souza et al., 2011). The results from molecular identification and genotyping of *C. neoformans* and *C. gattii* species complex can positively impact the monitoring of resistant strains and treating cryptococcosis (Sidrim et al., 2010).

This updated review sheds a light on the main molecular techniques for accurate identification, and typing of cryptococcosis primary agents from different environmental sources. Moreover, the susceptibility profiles of environmental isolates of various genotypes were also presented.

## ENVIRONMENTAL PREVALENCE

*Cryptococcus neoformans* and *C. gattii* are saprophytes and their infections occur by the exogenous route via inhalation of infectious dispersed yeast cells. Hence, awareness by *C. neoformans* and *C. gattii* environmental prevalence (Table 1) is critically important for laying out the possible control measurements against cryptococcosis (Chowdhary et al., 2011).

## IDENTIFICATION OF *C. NEOFORMANS*/*C. GATTII* SPECIES COMPLEX

The microbiology laboratory must differentiate *C. gattii* and *C. neoformans* as they differ in the susceptibility patterns to antifungal drugs. Only 5% of New York State surveyed laboratories had the ability of proper identification and differentiation of *C. gattii* and *C. neoformans*; while in developing countries, this issue is more unsatisfactory (Chowdhary et al., 2011). *Cryptococcus neoformans/C. gattii* species complex has been classically identified by phenotypic methods based on the species characteristics. Various molecular methods have been further applied for confirmation of the identification and also for genotyping purposes. The MALDI-TOF MS technique has been successfully used for identification and discrimination of this species complex (Perfect and Bicanic, 2015; Hagen et al., 2015).

## PHENOTYPIC METHODS

Phenotypically, *C. neoformans* and *C. gattii* were identified by different tests such as polysaccharide capsule production, which is detected with India ink staining, urease and phenoloxidase tests, and growth at 37°C. Cryptococcal species are maintained in the culture of solid media as Sabouraud dextrose agar medium with or without the addition of antibiotics after isolation. The opaque, and creamy to tan or brown colored colonies are fast-growing and observed after up to 3 days of incubation at 25°C and 37°C. Carotenoid pigments may be produced after a long incubation period. The colony slimy appearance is related to the capsule size (Doering, 2009). For *C. gattii* differentiation from *C. neoformans,* many phenotypic methods have been used including Canavanine Glycine Bromothymol blue agar (CGB), which may give false-positive results, Glycine Cycloheximide Phenol red agar, Creatinine-Dextrose Bromothymol blue agar, and Creatinine Dextrose Bromothymol blue thymine agar (Byrnes and Marr, 2011). Available commercial approaches

**Table 1:** Environmental prevalence of *Cryptococcus neoformans* and *Cryptococcus gattii* species complex globally.

| Type of samples (No.) | Prevalence (%) | | Country | Molecular type | Method of typing | Reference |
|---|---|---|---|---|---|---|
| | *C. neoformans* | *C. gattii* | | | | |
| Pigeon droppings (191) | 4.7 | - | Brazilian amazon | VNI | PCR-RFLP | Alves et al. (2016) |
| Trees hollows (pottery tree) (255) | - | 0.4 | | VGII | | |
| Pigeon droppings (50) | 10 | - | Mexico | C. neoformans var grubii | PCR and ITS1,ITS2 sequencing analysis | Canónico-González et al. (2013) |
| Cocktail and love bird excreta (200) | 7.5 | - | Egypt | serotype A | Multiplex PCR, ITS sequencing | Elhariri et al. (2015) |
| Eucalyptus trees sample (311) | 4.2 | - | Egypt | *C. neoformans var grubii* serotype A | Multiplex PCR | Elhariri et al. (2016) |
| Olive Trees (*Olea europaea*) (388) | 22.4 | - | coastal line of Anatolia, Turkey | Serotype A | Multiplex PCR, ITS sequencing | Ergin et al. (2019) |
| Passerine and Psittacine excreta (141) | 25.5 | - | Brazil | VNI | Multiplex PCR, PCR fingerprinting | Lugarini et al. (2008) |
| Pigeon dropping (100) | 32 | - | Libya | VNI | Multiplex PCR, *URA5* RFLP | Ellabib et al. (2016) |
| Eucalyptus trees (210) | 1.4 | - | | VNI | | |
| Hollows of trees and soil around trees (272) | - | 5.9 | California, USA | VGI | MLST | Hurst et al. (2019) |
| Olive tree hollows (172) | 12.2 | 1.1 | Italy | VNI | Multiplex PCR, MLST | Trovato et al. (2019) |
| *Castanopsis argyrophylla* trees hollows (48) | - | 2.08 | Thailand | VGI/ AFLP4 | AFLP genotyping and MLST | Khayhan et al. (2017) |
| *Olea europea* and *Pinus sylvestris* trees hollows (472) | 0.8 | - | Croatia | VNI/ VNIV | Multiplex PCR | Pllana-Hajdari et al. (2019) |

PCR-RFLP: PCR-restriction fragment length polymorphism, ITS: internal transcribed spacer, MLST: multi-locus sequence typing, AFLP: amplified fragment length polymorphism.

were used for yeast identification; for instance, API 20 AUX (bioMeriex, Paris, France) and VITEK 2 system (bioMérieux, Inc., Hazelwood, MO), which couldn't differentiate *C. gattii* from *C. neoformans*, while MALDI-TOF MS was successfully used for this differentiation (Mctaggart et al., 2011).

Limitations of conventional methods of identification and diagnosis led to many molecular methods development that were used for identification and genotyping of *C. neoformans* and *C. gattii* species complex (Cogliati, 2013).

## MOLECULAR METHODS FOR IDENTIFICATION AND GENOTYPING OF *C. NEOFORMANS* AND *C. GATTII* SPECIES COMPLEX

Molecular approaches are of great sensitivity and specificity, and could overcome the restrictions of traditional methods. It is employed for identification and typing of *C. neoformans/C. gattii* complex (Table 2) besides the molecular epidemiology research (Meyer, 2015).

Numerous molecular typing methodologies were already applied in the determination of subgroups of each species of *C. neoformans/ C. gattii* complex as multilocus enzyme electrophoresis (MLEE), random amplification of polymorphic DNA (RAPD), PCR fingerprinting (Cogliati, 2013), restriction fragment length polymorphism (RFLP) of phospholipase (*PLB1*), orotidine monophosphate pyrophosphorylase (*URA5*), and *GEF1* genes (Brito-Santos et al., 2015), and sequencing of internal transcribed spacer regions (ITS1 and ITS2) or intergenic spacer region (IGS), amplified fragment length polymorphism (AFLP) (Meyer et al., 2009). Recently, multilocus sequence typing (MLST) (Beale et al., 2015), multilocus microsatellite typing (MLMT), genes sequence analysis, whole-genome analyses, whole-genome sequencing in addition to MALDI-TOF MS analysis (D'Souza et al., 2011; Hagen et al., 2015) were introduced for genotyping.

## DNA–DNA HYBRIDIZATION METHODS AND ELECTRO-KARYOTYPING

DNA–DNA hybridization techniques and electro-karyotyping were used in a wide range in 1990 for cryptococcosis research with a special reference to its main causative agents. They were more useful and accurate when combined with PCR in the researches that are concerned with the most rapid and economical progressions of alternative technologies in *Cryptococcus* species identification (Hu et al., 2008). These methods are costly and complex as they need previous electrophoresis reliability, denaturing buffer preparation, nitrocellulose or nylon membrane acquiring for DNA impregnation, uniquely marked probes, and suitable detection equipment (Sidrim et al., 2010).

## PCR ASSAYS

These techniques are widely used in laboratories to detect *Cryptococcus* DNA and to genotype *C. neoformans* complex from clinical and also environmental samples as it is specific, fast, easily performed, and sensitive (Feng et al., 2013). Moreover, PCR assays are entirely automatic and able to discriminate yeasts from either clinical samples or contaminated cultures. PCR is used with more other techniques to be valuable for molecular epidemiology researches (Cogliati, 2013). The most commonly used PCR methods in identifying *C. neoformans* and *C. gattii* are nested, multiplex, and real-time targeting the orotidine monophosphate pyrophosphorylase (*URA5*) and the capsule synthesis (*CAP59*) genes, minisatellite-specific core sequence (M13), and ITS regions of rDNA (18S, 5.8S, and 28S) target sequences (Hagen et al., 2012) as following.

## NESTED PCR

Nested PCR is a greatly sensitive, rapid, and reliable approach for identifying *Cryptococcus* species and diagnosing cryptococcosis, in which the DNA template is a product of the first-round PCR. It is also a useful technique used for patients monitoring throughout the therapy and for confirmation of the fungal pathogen clearance in the follow-up examinations (Rivera et al., 2015). In 2002, *C. neoformans* was directly detected from laboratory animal tissue samples by 18S region of rDNA amplification (Bialek, 2005). Hyper-branched rolling circle amplification (HRCA) is a semi-nested PCR, which is based on PLB1, the padlock probes locus. This practice was highly sensitive and more specific with the ability of distinct nucleotide polymorphisms identification and it is used in the direct cryptococcosis diagnosis (Trilles et al., 2014).

**Table 2:** Molecular techniques used for identification and genotyping of *Cryptococcus neoformans/C. gattii* species complex.

| Technique | Target | Advantage | Disadvantage | References |
|---|---|---|---|---|
| **Identification** | | | | |
| Hybridization | Repeatable and polymorphic DNA | High specificity and sensitivity | Costly and laborious | Hu et al., (2008) |
| Nested PCR | ITS ribosomal DNA | High sensitivity and specificity | Presence of reaction contaminants<br>Presence of polymorphism | Rivera et al. (2015) |
| Multiplex PCR | Serotype specific | Amplification of two or more loci in just one reaction. Small amounts of DNA extracted | Reagent competition<br>Non-specific products | Leal et al. (2008) |
| Real-time PCR | 18S/28 ribosomal RNA | High sensitivity, specificity and speed<br>Determining levels of gene expression,<br>Fast | Contamination with genomic DNA<br>Requires technical ability and support<br>Expensive | Feng et al. (2013) |
| **Genotyping:** | | | | |
| PCR fingerprinting | Microsatellite (GACA)4 | Previous knowledge of target sequences is not required Using of short primers Detection of polymorphism | Standardization of the technique under the conditions of each laboratory | Meyer et al. (2009) |
| RAPD | Minisatellite (M13) | Previous knowledge of target sequences is not required Using of short primers Detection of polymorphism | Standardization of the technique under the conditions of each laboratory | Sidrim et al. (2010) |
| PCR-RFLP | Urease | Specificity | Decreased sensitivity in case of mutation | Cogliati (2013) |
| AFLP | Capsule | High sensitivity and specificity<br>Detection of genetic variability. | Large number of phases and reagents.<br>Expensive | Meyer et al. (2009) |
| MLST | IGS, capsule, laccase, urease, phospholipase | Reproducible and accurate.<br>Completely automated analysis.<br>Analysis of multiple loci | Restrictions in strains differentiation when genes are conserved | Beale et al. (2015) |

ITS: internal transcribed spacer, RAPD: random amplification of polymorphic DNA, PCR-RFLP: PCR-estriction fragment length polymorphism, AFLP: amplified fragment length polymorphism, MLST: multi-locus sequence typing, IGS: intergenic spacer region.

### MULTIPLEX PCR

Multiplex PCR is another desirable approach that enables the amplification of more loci in just one reaction. This technique is rapid, done with less amount of DNA, and species-specific. Moreover, it has been used to check the fungal isolates mating-type profile and it was applied in conjunction with other methods; for instance, the real-time PCR assay (Ito-Kuwa et al., 2007). Leal and his co-workers established a protocol of multiplex PCR using species-specific primers for ITS region. The obtained findings indicated a specific and rapid differentiation between *C. neoformans* and *C. gattii* from different isolates (Leal et al., 2008).

### REAL-TIME PCR

Real-time PCR, a recent method applied for rapid and accurate *C. neoformans* and *C. gattii* identification, allows the evaluation of gene expression associated with the microorganism virulence. This technique achieves higher levels of sensitivity, but it still of high cost, where expensive materials and special equipment are needed (Feng et al., 2013).

### PCR FINGERPRINTING

The major typing approach in *C. neoformans* comprehensive molecular epidemiological studies is PCR fingerprinting. It allowed the determination of molecular types in sporadic diseases and it was used in molecular epidemiology studies (Posteraro et al., 2012). This assay relies on DNA sequences amplification lined by single primers in PCR including primers for the minisatellite-specific core sequence of the wild-type phage M13 as well as microsatellite-specific single-primers $(GACA)_4$ (Meyer et al., 2009). PCR fingerprinting classified *C. neoformans* complex into eight main types depending on the polymorphic DNA sequences as following: *C. neoformans* var. *grubii* serotype A1 (VNI), *C. neoformans* var. *grubii* serotype A2 (VNII), *C. neoformans* serotype AD (VNIII), *C. neoformans* var. *neoformans* serotype D (VNIV), and *C. gattii* B and C serotypes (VGI, VGII, VGIII, and VGIV) (Perfect and Bicanic, 2015).

### PCR– RFLP

PCR– RFLP using the *URA5* and *PLB*1 genes was previously used in *C. neoformans* complex molecular types confirmation (Brito-Santos et al., 2015). This assay was applied in molecular epidemiological surveys not only for evaluation of the possible relationships between clinical and environmental *C. neoformans* complex molecular types, but also for cryptococcosis diagnosis as it commonly targets the *URA5* gene (Kwon-Chung et al., 2017). This practice is also suggested when it is desired to get more information about a particular strain. Moreover, other targets rather than *URA5* were used for molecular typing and accurate serotypes differentiation such as the capsular gene, *CAP59* (Feng et al., 2013).

### RAPD ASSAY

RAPD analysis had been used for investigation of the genetic variability of *Cryptococcus* spp. from different sources and also for determination of serotypes and molecular types. It is rapid, simple, and highly discriminatory requiring strict quality control measures, but it is recently replaced by more recent techniques for *C. neoformans* and *C. gattii* genotyping (Sidrim et al., 2010).

### AFLP ASSAY

AFLP is another useful technique in *C. neoformans* complex genotyping. It is reliable, more specific than RAPD due to using of longer primers in the PCR cycles that prevent the existence of conflict during the PCR reaction of the RAPD technique (Meyer, 2015). The AFLP technique stages are DNA cleavage using *Eco*RI restriction enzyme and rarely *Mse*I, then ligation of particular adaptors to the ends of DNA fragments, PCR cycles using *Mse*I-G and *Eco*RI-AC primers, and finally high-resolution gel electrophoresis. The large numbers of stages, reagents, and devices as well as DNA quality are considered limitations of this technique (Sidrim et al., 2010). The AFLP assay exposed *C. neoformans* and *C. gattii* strains to subdivision into 7 - 9 genetically diverse monophyletic clades (Kwon-Chung et al., 2017). The AFLP assay has helped to clarify the Vancouver island outbreak causative agent due to its high discriminatory power besides the ability to give a particular distinctive profile to each strain; the outbreak agent was two AFLP6 subtypes (AFLP6A and AFLP6B) (Byrnes and Marr, 2011). Some studies in the Netherlands applied the AFLP typing technique to *Cryptococcus* strains and discovered novel hybrids among *C. neoformans* and *C. gattii*, AFLP9 (AFLP1×AFLP4) and AFLP8 (AFLP3×AFLP4) (Hagen et al., 2012).

### MULTI-LOCUS SEQUENCE TYPING

Multi-locus sequence typing has significant importance for the global epidemiological characterization of *Cryptococcus* species complex genotypes around the world (Firacative et al., 2016). It exploits the unique characteristics of nucleotide sequences of multiple genes such as capsular, urease, and phospholipase encoding genes for allowing *Cryptococcus* spp typing (Beale et al., 2015). MLST analysis is fully automated after the target regions amplification and sequencing and it can be interconnected between laboratories (Meyer, 2015). A limitation of this technique is that it provides incomplete or inaccurate measures of the species relationships as a relatively small portion of sequence diversity is detected by MLST and also it yields weak recombination interpretations (Beale et al., 2015).

In 2007, International Society of Human and Animal Mycoses developed and sponsored a workgroup that declared MLST as a standard method for *C. neoformans - C. gattii* genotyping (Firacative et al., 2016). Typical MLST involves 7 loci sequences: capsular-associated protein (*CAP*59), glyceraldehyde-3-phosphate dehydrogenase (GPD1), *URA*5, *PLB*1, laccase (*LAC*1), Cu, Zn superoxide dismutase (SOD1), and intergenic spacer region (IGS1). These sequences together represent the minimal genes numbers that give the maximum power of discrimination (Farrer et al., 2015).

The recent proposed designation for *C. neoformans/C. gattii* species complex depending on AFLP and MLST results is as follows: I. *C. neoformans* serotype A (VNI) AFLP1, VNII AFLP1A, AFLP1B, VNB, II. *C. deneoformans* serotype D (VNIV) AFLP2, and III. *C. neoformans - C. deneoformans* hybrid or AD hybrids (VNIII) AFLP3. *Cryptococcus gattii* has been categorized as separate species named *C. gattii* (VGI) AFLP4, *C. deuterogattii* (VGII) AFLP6, *C. bacillisporus* (VGIII) AFLP5, *C. tetragattii* (VGIV) AFLP7, and *C. decagattii* (VGIV) and (VGIIIc) AFLP10. The hybrids between *C. neoformans* and *C. gattii* complexes isolates are named *C. deneoformans* × *C. gattii* hybrid (AFLP8), *C. neoformans* × *C. gattii* hybrid (AFLP9), and *C. neoformans* × *C. deuterogattii* hybrid (AFLP11) (Hagen et al., 2015). The expression "*neoformans*" was used for long time to represent species and variety. *C. neoformans* var. *neoformans* in that new system "*C. neoformans*" represents only serotype A strains with VNI and VNII/VNB molecular types (Kwon-Chung et al., 2017).

## MULTI-LOCUS MICROSATELLITE TYPING

Multi-locus microsatellite typing (MLMT) has been commonly used for typing of different fungi owing to its strong discriminatory power, which might be an effective and provable approach for wide-range epidemiology studies (Feng et al., 2013). The MLMT with MLST were applied for studying the genotypic diversity and genetic relationships between environmental and clinical isolates of *C. neoformans* in an Indian survey, where environmental isolates showed more genetic diversity than clinical ones (Prakash et al., 2020).

## WHOLE GENOME SEQUENCING

The whole fungal genome sequencing technique is an accurate and modern technique for typing of *Cryptococcus* strains (D'Souza et al., 2011). Genes` contents and structure characterization indicated variations in the *Cryptococcus* genes in comparison with other fungi as *Cryptococcus* genes are intron-rich with predicted highly alternative splicing and antisense transcription (Janbon et al., 2014). The whole genome structure is designed to be significantly heterogeneous within *Cryptococcus* strains with few alterations of either species or molecular types (Farrer et al., 2015). *Cryptococcus neoformans* var. *grubii* and *C. neoformans* var. *neoformans* genomes showed extensive rearrangements (Janbon et al., 2014).

## MATRIX-ASSISTED LASER DESORPTION IONIZATION-TIME OF FLIGHT MASS SPECTROMETRY

The MALDI-TOF MS is a simple, fast, and accurate technique based on mass spectrometry of different microorganisms. It was used as an alternative for phenotypic and genotypic methods for *C. neoformans* and *C. gattii* differentiation (Firacative et al., 2012; Hagen et al., 2015). This technique allows the discrimination between species by evaluation of the determined spectrum of peptides and proteins of integral microbial cells either from biological samples or uncontaminated cultures within few minutes and it also separates *C. neoformans* and *C. gattii* into eight molecular patterns (Firacative et al., 2012).

## ANTIFUNGAL SUSCEPTIBILITY TESTING

The best antifungal drug should target either a component vital for the viability of fungal cell or a virulence factor, but not the host to avoid cell toxicity. That drug should have a fungicidal effect when used alone or in combination with another. It also should be of good bioavailability and capable of reaching cryptococcal receptors within the host (May et al., 2016). The World Health Organization and the Infectious Diseases Society of America recommended the gold standard cryptococcosis therapy guidelines including three drugs: amphotericin B (AmB), flucytosine, and fluconazole (Maziarz and Perfect, 2016).

Amphotericin B deoxycholate, which was released in 1960, acts as fungicidal by binding to the ergosterol of fungal cell membrane and also by cell death induction as a result of oxidative damage (Gray et al., 2012). Using AmB prevailing formulations requires the intravenous route and its usage is difficult in oral administration because of low bioavailability (Kwon-Chung et al., 2014).

Flucytosine is converted inside the fungal cells principally by the cytosine deaminase enzyme to 5-fluorouracil (5-FU). The mammalian cells are lacking this enzyme (Loyse et al., 2013). Fluconazole has a good bioavailability, essentially in the initial stage therapy and it may be used during the maintenance stage. It acts as a fungistatic (rather than fungicidal) as it inhibits the ergosterol synthesis leading to accumulation of destructive steroidal substances in the cell membrane (Gray et al., 2012).

Using the standard antifungal susceptibility testing methods has facilitated the detection of antifungal resistance; for example, clinical breakpoints and epidemiologic cut off values for *Candida* and *Aspergillus* spp., the Clinical and

**Table 3:** Antifungal susceptibility profiles of *Cryptococcus neoformans* and *C. gattii* from environmental samples according to Clinical Laboratory Standards Institute guidelines.

| **Species (No.)** | **Geographic region** | **MIC range (µg/mL)** | | | | | | | | | **Reference** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **AMB** | **FLU** | **5-FC** | **VRC** | **KET** | **ITC** | **POS** | **ISA** | **Nys** | |
| *C. neoformans* (10) | Cameroon | 4-8 | 64- >256 | ND | ND | 16-64 | ND | ND | ND | 0.125-0.5 | Dongmo et al. (2016) |
| *C. gattii* (10) | | 64-128 | 16- >256 | ND | ND | 8-64 | ND | ND | ND | 0.5-1 | |
| *C. neoformans* (86) | India | 0.062-0.5 | 2-8 | 1-16 | 0.031-0.250 | ND | 0.031-0.5 | ND | ND | ND | Chowdhary et al. (2011) |
| *C. gattii* (60) | | 0.125-1 | 2-16 | 1-4 | 0.062-0.250 | ND | 0.125-0.5 | ND | ND | ND | |
| *C. neoformans* (117) | India | 0.004-0.25 | 0.032-12 | ND | 0.006-0.125 | 0.002-0.19 | 0.004-0.75 | ND | ND | ND | Khan et al. (2007)* |
| *C. gattii* (65) | | 0.023-0.5 | 0.032-16 | ND | 0.004-0.125 | 0.003-0.19 | 0.006-2 | ND | ND | ND | |
| *C. neoformans* (81) | Brazil | 0.3-1 | 0.12-64 | ND | 0.3-2 | 0.3-2 | 0.3-1 | ND | ND | ND | Andrade-Silva et al. (2013) |
| *C. neoformans* (40) | Brazil | 0.15-0.125 | 0.25-2 | ND | 0.003-0.25 | ND | 0.007-0.125 | ND | ND | ND | Souza et al. (2005) |
| *C. neoformans* (40) | Goiania, Brazil | 0.03-0.5 | 0.5-4 | ND | 0.03-0.25 | ND | 0.03-0.125 | ND | ND | ND | Souza et al. (2010) |
| *C. neoformans* (16) | Sao Paulo, Brazil | 0.25-2 | 0.5- >64 | 1- >64 | ND | ND | 0.06-4 | ND | ND | ND | Pedroso et al. (2006) |
| *C. gattii* (60) | North western India | 0.6-1 | 0.25-16 | 0.125-64 | 0.3-0.25 | 0.016>-0.5 | ND | 0.016>-0.5 | 0.016>-0.25 | ND | Chowdhary et al. (2013) |
| *C. neoformans* (50) | India | ND | 0.063-64 | ND | ND | 0.03-0.25 | 0.03-1 | ND | ND | ND | Gutch et al. (2015) |
| *C. gattii* (4) | | ND | 2-64 | ND | ND | 0.03-0.125 | 0.03-0.5 | ND | ND | ND | |
| *C. neoformans* (8) | Brazil | ≤0.031–0.5 | ≤0.125–8 | 1-8 | ND | ND | ≤0.031–0.125 | ND | ND | ND | Moraes et al. (2003) |
| *C. gattii* (6) | | ≤0.031–0.125 | 0.125–8 | ≤0.125–8 | ND | ND | 0.5–2 | ND | ND | ND | |

MIC: minimum inhibitory concentration. No.: number of cryptococcal species, AMB: amphotericin B, FLU: fluconazole, 5-FC: 5-flucytocine, VRC: voriconazole, KET: ketoconazole, ITC: itraconazole, POS: posaconazole, ISA: isavuconazole, Nys: nystatin. ND: not determined, *: Antifungal sensitivity was performed using E-Test method.

Laboratory Standards Institute (CLSI) and Antifungal Susceptibility Testing of the European Committee on Antibiotic Susceptibility Testing (Pfaller, et al., 2011). The sensitivity of *C. neoformans* and *C. gattii* to antifungal agents have been determined using broth micro-dilution and macro-dilution methods according to the CLSI guidelines (Moraes et al., 2003) in addition to E-test (Khan et al., 2007).

Antifungal susceptibility testing helps in preference of the efficient antifungal therapies for immunocompromised patients with disseminated mycosis (Andrade-Silva et al., 2013). Studying the antifungal susceptibility patterns of *C. neoformans* and *C. gattii* environmental isolates (Table 3) is limited (Pedroso et al., 2006). Several studies revealed relatively low minimum inhibitory concentrations (MICs) of typical antifungals to *C. gattii* in contrast to *C. neoformans* with no increase over time (Thompson et al., 2008). In the past two decades, the reports of fluconazole-resistant strains increased globally. Geographical data indicates that increased fluconazole resistance was reported in Asia, Africa, and Latin America; while in North America, Europe, and Spain, low resistance rates were reported (May et al., 2016).

Many published studies stated that *C. gattii* and *C. neoformans* origins and genotypes had a great impact on the susceptibility to antifungal agents (Chong et al., 2010). In contrast, other investigators observed that susceptibility to antifungal agents was not influenced by the environmental or clinical origins of *Cryptococcus* species (Moraes et al., 2003).

The high prevalence and severity of *cryptococcal* infections are being considered as significant public health issues as a result of the high expansion in immunocompromised patients. Accordingly, using antifungals, principally in long-term therapies led to the resistance of *C. neoformans* and *C. gattii* species (Yang et al., 2010).

## CONCLUSIONS AND RECOMMENDATIONS

In conclusion, introducing molecular methods for accurate and rapid identification of *C. neoformans* species complex is warranted in the mycology laboratories for efficient monitoring of resistant strains and treating cryptococcosis. This updated review threw more light on the global prevalence, genetic diversity, ecology, and susceptibility profiles of the environmental isolates of *C. neoformans* species complex to investigate the severity of health hazards generated by *Cryptococcus* species and to design possible control measures against cryptococcosis.

## AUTHOR'S CONTRIBUTION

MT and YHT contributed to the conception and design of the article and participated with SZI and RMA in interpreting the relevant literature. SZI and RMA drafted the manuscript. YHT and MT revised it critically for important intellectual content.

## CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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