Amplification of Capsule-associated Genes from *Cryptococcus neoformans*

Randa Alarousy[*1], Heidy Abo El Yazeed[2], Hosam Kotb[3], Khaled Abdalla[4], and Mohamed Refai[2]

[1]Department of Microbiology and Immunology, National Research Centre, Giza, Egypt
[2]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[3]Department of Reproductive Diseases, Animal Reproduction Research Institute
[4]Department of Plant Molecular Biology, Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt

Abstract: The capsule of *C. neoformans* is an important virulence factor and its strains exhibit variability in their capsular polysaccharide, cell morphology and virulence, but the relationship between these variables is not well studied. In this study, four different phenotypes were under investigation, two vary in capsule diameter and another two vary in colony color and cell form, R, T, P and H phenotypes respectively. The phenotypic switching was induced by certain volatile oils (rosemary and thyme). Capsule-associated genes, CAP 10, CAP 59, CAP 60, and CAP 64 were amplified to explore the genetic profile of these genes. Agarose gel electrophoresis analysis of the PCR products revealed the same expected product for each gene from all phenotypes.

[Randa Alarousy, Heidy Abo El Yazeed, Hosam Kotb, Khaled Abdalla, and Mohamed Refai **Amplification of Capsule-associated Genes from *Cryptococcus neoformans***] New York Science Journal 2011;4(11):38-42]. (ISSN: 1554-0200). http://www.sciencepub.net/newyork.

Key words: *Cryptococcus neoformans*, Virulence, Phenotypes, Rosemary oil, Thyme oil

1. Introduction:

Cryptococcus neoformans is encapsulated yeast that causes many forms of cryptococcosis in man and in various animal species (1, 2). The organism is largely an opportunistic pathogen but can also be a primary pathogen in a small cohort of patients with no apparent immune system defects. Several factors have been identified to be required for *C. neoformans* virulence (1, 3). Factors that contribute to the virulence of the fungus are mainly related to its ability to grow at 37 ºC, to produce a thick polysaccharide capsule, release soluble products into the bloodstream to synthesize melanin and to be an alpha–mating phenotype (MAT alpha). The polysaccharide capsule is a major virulence factor of *C. neoformans.* The capsule interferes with phagocytosis, blocks the recruitment of inflammatory cells, increases co-stimulatory molecules, suppresses the delayed-type-hypersensitivity response, and reduces the antibody production in response to fungal infection (4). Fries *et al.* defined and used the phrase "phenotypic switching" to describe the process by which colony variants arise in a *C. neoformans* population that can revert to their original appearance at a switching frequency that is greater than the background mutation rate, it plays a crucial role in pathogenesis, as it facilitates dissemination to the CNS. Moreover, phenotypic switching enables pathogens to undergo rapid microevolution and to adapt to different microenvironments (5). Microevolution produces phenotypic variability that can alter the virulence of the pathogen. Gupta and Fries discussed how phenotypic traits in *Cryptococcus* species can vary and how the variability affects the pathogenesis of chronic cryptococcosis. Global changes of phenotypic traits occur in the whole fungal population under certain conditions. They studied some of these changes such as; capsule induction, melanization, biofilm formation and hyphal/spore formation (6). Capsule of *C. neoformans* cells exhibits a considerable degree of plasticity in its ability to undergo reversible changes. Many researches studied the induction of phenotypic switching in cryptococcal capsules, including the previous studies examined the effect of certain oils as rosemary, thyme and camphor oils in changing the capsule size of *C. neoformans* (7). The mechanism responsible for phenotypic switching is not yet known (8). In an attempt to understand the genetic base of microevolution, significant progress has been made in dissecting the biosynthetic pathways, as well as in characterization of the genes involved in capsule formation. Four genes CAP59, CAP64, CAP60, and CAP10 were identified by similar approaches to be required for the formation of capsule (9, 10, 11). The biochemical function and the role of these genes have to be elucidated. The aim of the present study was to reveal phenotypic changes using certain volatile oils and other environmental factors in addition to the amplification of capsule associated genes to explore the relationship between genotypic and phenotypic diversity in *C. neoformans.*

2. Materials and Methods:

2.1. Strains and growth conditions:

Phenotype O (original normal capsule): the standard strains of *C. neoformans* var. *grubii* serotype A (2041), and serotype C. These strains were maintained on Sabouraud Dextrose Agar (40.0 gm Dextrose; 10.0 gm peptone; 20.0 gm agar and 50.0 mg chloramphenicol per liter).

2.2. Induction of phenotypic switching using oils:

Phenotype R (large capsule): it was obtained by growing serotype A on Sabouraud Dextrose Agar containing 10% rosemary oil.

Phenotype T (small capsule): it was obtained by growing serotype A on Sabouraud Dextrose Agar containing 10% thyme oil.

2.3. Hyphal form induction:

Phenotype H was obtained by keeping *C. neoformans*, var. *grubii* serotype A (2041) lyophilized for prolonged period which lead to change to hyphal form and lost its capsule.

2.4. Pink pigmented phenotype:

Phenotype P: local strain (K 18) was kindly provided by Shimaa Abou-Elmagd (Directorate of Veterinary Medicine, Qena, Egypt); the strain was isolated from chicken droppings. This strain produced pink-pigmented colonies. All plates were incubated at 30 ºC for 5 or 6 days.

2.5. Characterization of capsule size and shed GXM:

Capsule size of the examined phenotypes (O, R, T, P and H) was measured from 24-h cultures in SD broth (40.0 gm Dextrose; 10.0 gm peptone and 50.0 mg chloramphenicol per liter). Yeast cells were suspended in India ink (Fisher, USA) and visualized at a magnification of ×1,000 with an Olympus AX70 microscope. Images were captured with a digital camera. Capsule measurements were made with 10 randomly chosen cells from each strain, using Adobe Photoshop 7.0 for Windows, and the capsule thickness was calculated using the conversion of 45 pixels to 1 micrometer (12). Determination of glucuronoxylomannan contents of the capsule using Phenol-sulfuric acid method according to Dubois *et al*., in brief, equal volumes of sample solutions from each phenotype and phenol solution (500 µl each) were mixed in a glass tube to which 2.5 ml of sulfuric acid reagent was rapidly added and vortexed. After incubation in a dark cabinet for 1 hour, a spectrometric analysis at wavelength 490 nm was performed. The amount of polysaccharides produced was calculated from a standard curve prepared with known concentrations (10-100 µg/ ml) of D-glucose (13).

2.6. Primers:

PCR primers were designed using DNA STAR software for Windows based on previously published sequences of capsule genes with default parameters. Primers for CAP59 gene were synthesized according to Nakamura *et al*. (14). Table (1) shows primer sequences, annealing temperature and expected product size. All primers were synthesized by Pioneer co., Korea.

Table (1): Primers used in this study

Primer	Sequence 5`-3`	Ta	Expected size
CAP10F	5`-TGCCCGAAGCCGTTGCTGAG-3`	57 ºC	474 bp
CAP10R	5`-ACCCGGAAACCCTTGGCACG-3`		
CAP59F	5`-GAGTGTCTCCGCAACCCGCA-3`	52 ºC	597 bp
CAP59R	5`-CCTACTCTGCCAAATCAACTC-3`		
CAP60F	5`-GCAGCGGCTTGCCATTCGTG-3`	48 ºC	603 bp
CAP60R	5`-AGTCCGTGGAGGCGTGGTCA-3`		
CAP64F	5`-ACCCTACCGGCCACGGACTC-3`	50 ºC	406 bp
CAP64R	5`-GGGCACCTTGATGGCTCGCA-3`		

Ta: annealing Temperature

2.7. PCR Amplification:

DNA was extracted using Analytikjena Bio Solution DNA purification kit, Germany, according to the manufacturer's instructions. Three µl of genomic DNA (30 ng) was used in PCR reaction to amplify each fragment in a final volume of 25 µl containing 20 pmol of each primer, 2.5 µl 10X buffer, 200 µM dNTPs and 2.5 units Taq polymerase (Fermentas, Germany). PCR was carried out in DNA Thermal cycler 9600 (Applied Biosystems, USA) by an initial 5 min denaturing at 94 ºC followed by 35 cycles of PCR. Each cycle

included 1 min of denaturation at 94 ºC followed by 1 min of annealing at 52 ºC for CAP 59; 50 ºC for CAP 64; 48 ºC for CAP 60 and 57 ºC for CAP 10 genes, and 2 min of polymerization at 72 ºC. The last cycle of PCR included an additional 5 min extension at 72 ºC. The reactions were analyzed by electrophoresis on 1.5 % agarose gel and the PCR products were detected by UV transilluminator.

3. Results:

3.1. Macroscopic characterization:

As shown in Fig.1A the colonies of the standard strains of *C. neoformans*, irrespective of its capsular size (phenotypes O, R and T) were creamy, opaque and moist, while colonies of phenotype H were greyish white dry serrated, wrinkled, and firmly attached to the agar surface (Fig.1B). However, phenotype P demonstrated pink-pigmented moist colonies (Fig.1C).

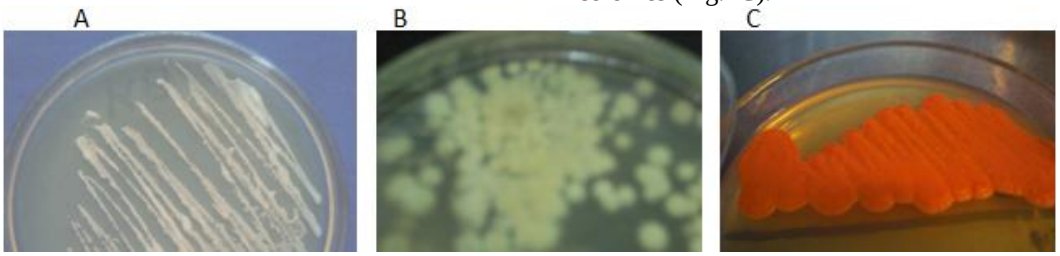

Fig.(1): Colonies morphology. A: Macroscopic morphology of original form phenotype O. B: Hyphal form of phenotype H. C: Pink-pigmented colonies of phenotype P. All phenotypes were grown on SDA media.

3.2. Microscopic morphology:

A halo of unstained capsule around the yeast cell was observed when using India ink preparation for phenotypes O, R, and T (Fig. 2 A, B and C respectively). As shown in Table (2) rosemary oil induced the biggest capsule size (0.35um) for phenotype R followed by the original phenotype O (0.19 µm) then phenotype P (0.12 µm) and finally thyme oil induced the smallest capsule size (0.094 µm) in phenotype T. hyphal form in phenotype H contains no capsule.

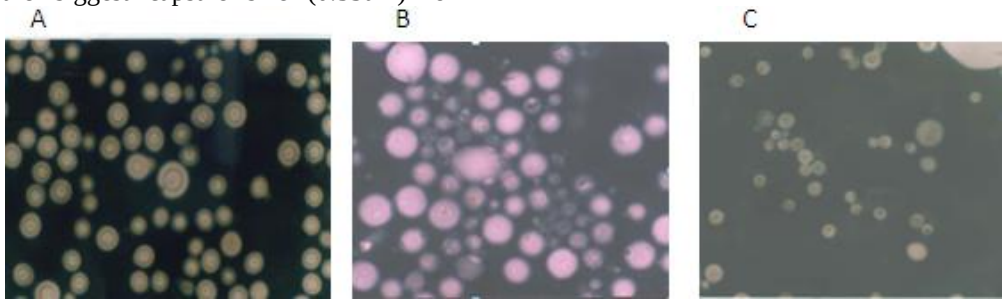

Fig.(2): Microscopic morphology. A: Capsule is demonstrated by light micrographs of phenotype O grown on SDA medium. B: Cells of phenotype R grown on rosemary oil agar. C: Cells of phenotype T grown on thyme oil agar media. All were stained with India ink and the capsule appears as a region of ink exclusion surrounding the cell.

Table (2): Capsule diameter averages and capsular GXM of examined *C. neoformans* phenotypes.

Phenotype	Inducer	Capsular diameter	GXM content
Phenotype O	Original	0.19 µm	2.4 mg/ml
Phenotype R	Rosemary oil	0.35 µm	6.11 mg/ml
Phenotype T	Thyme oil	0.094 µm	0.2 mg/ml
Phenotype P	Original	0.12 µm	1.8 mg/ml
Phenotype H	Lyophilizing	No capsule	No capsule

3.3. Detection of capsule-associated genes:

PCR amplification was undertaken for specific target sequences encoding CAP 10, CAP59, CAP60 and CAP64 genes with specific primers for each gene. All PCR reactions were performed for the tested strains (A and C) and phenotypes (P, H, T and R). The amplification of CAP 10 gene using CAP 10 primers revealed the same PCR product (~ 474 bp) for each strain as indicated in (Fig. 3A). As shown in (Fig. 3B), PCR product of ~ 597 bp were amplified for

CAP 59 gene while the amplification of CAP 60 gene revealed ~ 603 bp (Fig. 3C). However, PCR product of ~ 406 bp was revealed for the amplification of capsule gene CAP 64 (Fig. 3D). All six strains showed the same patterns and sizes of the capsule-associated genes.

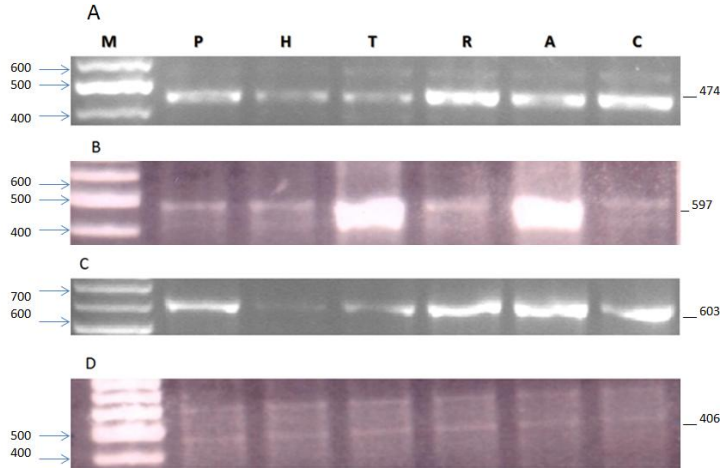

Fig. (3): PCR amplifications of capsule-associated genes. M: 100 bp Ladder marker. Lanes (P, H, T, R A and C), PCR products from *C. neoformans* phenotypes P, H, T, R, serotypes A and C respectively. A: Amplification of CAP 10 gene (~ 474 bp). B: Amplification of CAP 59 gene (~ 597 bp). C: Amplification of CAP 60 gene (~ 603 bp). D: Amplification of CAP 64 gene (~ 406 bp)

4. Discussion

Cryptococcus neoformans is an encapsulated yeast that belongs to the phylum Basidiomycota. The most distinctive virulence determinant of *C. neoformans* is the polysaccharide capsule; the major component of this capsule is a highly acidic, linear polysaccharide termed glucuronoxylomannan (15, 16). The capsule may affected by many environmental factors causing diversity or phenotypic switching. Many attempts were applied to understand the mechanism of phenotypic switching. In the current study, phenotypic switching was induced by certain oils, data illustrated in (Fig. 2) confirmed the role of these oils on the polysaccharide capsule of *C. neoformans* as confirmed previously (7). Data revealed that certain oils, such as rosemary increased capsule size of *C. neoformans* (Fig. 2B), while other such as thyme oil, decreased its capsule size (Fig. 2C) in comparison to the original capsule size of phenotype O (Fig. 2A). The mechanism by which capsule responds to the effect of certain oils was not explained until now. However, Poeta (17) suggested that changes in capsule structure may be related to responses to specific environmental conditions, with important implications for the host immune response. The characterization of capsules of the examined phenotypes was performed via micrographical measuring of the capsule diameter and chemical determination of capsular GXM content. Results showed in Table (1) revealed that GXM contents were directly proportional to the capsular diameter; this result is strongly supported by that of Yoneda and Doering (18), where their study revealed that modification in GXM molecules plays a central role in determining *C. neoformans* capsule size. However, Pierini and Doering (19) referred the diversity of GXM to some physiological settings such as alteration in growth conditions that affecting polysaccharide branching and overall capsule density. Change in capsular size was not the only phenotypic switching parameter investigated during this study, another two phenotypic parameters were also studied; pink–pigmentation of the yeast colonies (Fig.1C) and the growth of *C. neoformans* in the perfect hyphal form (Fig.1B). During our study, we detected wrinkled, pseudohyphal form of the yeast. Similar finding were detected by Jain *et al.* (20) where they concluded that phenotypic switching character exhibits differences in colony morphology. The phenotype H also reverted to its wild, yeast form, or imperfect form of *C. neoformans* but through their passage experimentally in mice (7), and this in agreement with Lin and Heitman (21). While phenotype P was the only one which did not revert to its wild phenotypic character, it did not switch from pink colored colonies to creamy colored colonies (Fig.1A), and this may be referred to colony color character of this phenotype. Moreover, this finding is supported by results of Fell and Phaff (22); Phaff and Fell (23); and Saez and Rodrigues de Miranda [24] who reported that there are many species and strains of *Cryptococcus* that may be characterized by pink–colored colonies. It was important to determine the genetic background of phenotypic switching through

amplification of the capsular genes, namely CAP 59; CAP 60; CAP 64 and CAP10. Results showed that there was not any variability or difference in the PCR products of the examined phenotypes. This identity indicated that a phenotypic variation in capsule size in *C. neoformans* strains and phenotypes was not a result of genetic variation and these findings agreed with those found by Small *et al.* (25). In summary, we characterized in this study the phenotypic switching due to the effect of certain oils. Our results demonstrate the use of these oils to change capsule size and that could lead to alteration of the virulence of *C. neoformans* and thus help in the treatment of *C. neoformans* infection. The potential contribution of the phenotypic switching is an interesting topic for future investigation.

Corresponding author
Randa Alarousy
Department of Microbiology and Immunology,
National Research Centre, Giza, Egypt
randaarousi@yahoo.com

References:

1. Casadevall, A. and J.R. Perfect. 1998. *Cryptococcus neoformans*, ASM Press, Washington, D.C. pp:147–151
2. Lengeler, K.B. and J. Heitman. 2002. *C. neoformans* as a model fungal pathogen. In Molecular Biology of Fungal Development, ed. H.D. Osiewacz, New York: Dekker. pp:513-57.
3. Steenbergen, J.N. and A. Casadevall. 2003. The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. Microbes. Infect. 5:667-675.
4. Vecchiarelli, A., D. Pietrella, P. Lupo, F. Bistoni, D.C. McFadden and A. Casadevall. 2003. The polysaccharide capsule of *Cryptococcus neoformans* interferes with human dendritic cell maturation and activation. J. Leukoc. Biol. 74:370-378.
5. Fries, B.C., C.P. Taborda, E. Serfass and A. Casadevall. 2001. Phenotypic switching of *Cryptococcus neoformans* occurs and influences the outcome of infection. J. Clin. Investig. 108:1639-1648.
6. Fries, B.C., D.L. Goldman and A. Casadevall. 2002. Phenotypic switching in *Cryptococcus neoformans*. Microbes Infect. 4:1345-1352.
7. Alarousy, R.M. 2004. Molecular biology studies on *Cryptococcus neoformans*. M. V. Sc. Thesis. Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.
8. Bose, I., A.J. Reese, J.J. Ory, G. Janbon and T.L. Doering. 2003. A yeast under cover: the capsule of *Cryptococcus neoformans*. Eukaryot. Cell. 2:655-663.
9. Chang, Y.C., L.A. Penoyer. and J. Kwon-Chung. 1996. The second capsule gene of *Cryptococcus neoformans*, CAP64, is essential for virulence. Infect. Immun. 64:1977–1983.
10. Chang, Y.C. and K.J. Kwon-Chung. 1998. Isolation of the third capsule-associated gene, CAP60, required for virulence in *Cryptococcus neoformans*. Infect. Immun. 66:2230–2236.
11. Chang, Y.C., and K.J. Kwon-Chung. 1999. Isolation, characterization, and localization of a capsule-associated gene, CAP 10, of *Cryptococcus neoformans*. J. Bacteriol. 181:5636–5643.
12. Zaragoza, O., B.C. Fries and A. Casadevall. 2003. Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO2. Infec. Imm. 71(11):6155–6164.
13. Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method of determination of sugar and related substances. Anal. Chem. 18:350-356.
14. Nakamura, Y., R. Kano, S. Watanabe, and A. Hasegawa. 2000. Molecular analysis of CAP59 gene sequences from five serotypes of *Cryptococcus neoformans*. J. Clin. Microbiol. 38 (3):992-995.
15. Doering, T.L. 2009. How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in *Cryptococcus neoformans*. Ann. Rev. Microbiol. 63:223-247.
16. Janbon, G. 2004. *Cryptococcus neoformans* capsule biosynthesis and regulation. FEMS Yeast Res. 4:765-771.
17. Poeta, D.M. 2004. Role of Phagocytosis in the Virulence of *Cryptococcus neoformans*. Eukaryot Cell. 3:1067-1075.
18. Yoneda, A., T.L. Doering. 2008. Regulation of *Cryptococcus neoformans* capsule size is mediated at the polymer level. Eukaryot. Cell. 7:546–549.
19. Pierini, L.M., and T.L. Doering. 2001. Spatial and temporal sequence of capsule construction in *Cryptococcus neoformans*. Mol. Microbiol. 41:105–115.
20. Jain, N., F. Hasan and B.C. Fries. 2008. Phenotypic Switching in Fungi. Curr. Fungal Infect. Rep. 2(3):180–188.
21. Lin, X. and J. Heitman. 2005. Chlamydospore Formation during Hyphal Growth in *Cryptococcus neoformans*. Eukaryotic Cell. 4(10):1746-1754.
22. Fell, J.W. and H.J. Phaff. 1967. In The yeasts, a taxonomic study, third revised and enlarged edition. El sevier Science. Publishers. B. V. Amsterdam.
23. Phaff, H.J. and J.W. Fell. 1970. In The yeasts, a taxonomic study, third revised and enlarged edition. El sevier Science. Publishers. B. V. Amsterdam.
24. Saez, H. and L. Rodrigues de Miranda. 1988. In The yeasts, a taxonomic study, third revised and enlarged edition. El sevier Science. Publishers. B. V. Amsterdam.
25. Small, J.M., T.G. Mitchell and R.W. Wheat. 1986. Strain variation in composition and molecular size of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. Infect. Immun. 54:735-741