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Determination of Mating Types through Antibiotic Resistance Phenotypes in *Cryptococcus neoformans*

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ABSTRACT: *Cryptococcus neoformans* is best known as a life threatening pathogen that infest majorly immuocompromised individual. In this work, two isolates; one clinical CD4832 and environmental NMB5 selected and confirmed based on their melanin production on niger seed agar and ability to grow at 37°C. These selected isolates were involved in antifungal susceptibility test using Fluconazole as the antibiotic agent. Response to Fluconazole showed that the CD4832 is less susceptible compare to NMB5. The results clearly demonstrated that the two strains have different susceptibility levels based on the various concentration of the Fluconazole that was used. In attempting to check if the two strains have the ability to recombine, they were mixed together and treated with the same concentration of Fluconazole. Interestingly, the results suggested two mating types- thus opening wide discussion for research into new drug target.

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Cryptococcus neoformans are the main causative agents of Cryptococcoccosis. Cryptococcus is a basidiomycetes yeast that causes infections after inhalation of its basidiospores found in the environment (Martinez and Casidevall, 2006; Giles *et al*., 2009; Negroni, 2012). The clinical manifestation usually occur in cerebral and pulmonary forms and then may progress to the most severe form of the disease, meningeoncephalitis (Chau *et al.,* 2010; Kester *et al.,* 2010; Negroni, 2012). *C. neoformans* is estimated to be the cause of more than a million cases of crytococeosis every year with about 625,000 deaths worldwide (Park *et al*., 2009). An estimate of 720,000 cases are reported to occur in sub-Saharan Africa (Park *et al*., 2002) and an estimate of 500,000 deaths annually.

Two types of mating in *C. neoformans* are recognized, mating types α and a (Kwon-Chung, 1976). More than 95% of all clinical and environmental isolates of *C. neoformans* are Mat α Serotype A isolates (Aα) (Halliday *et al*., 1999; Klickes *et al*., 1996; Yan *et al*., 2002). The bias in this mating type ratio has been postulated to be as a result of the wild type haploid MAT α cells that under suitable conditions develop a hyphal phase, producing basidia with viable basidiospores (Kwon-Chung *et al*., 1992; Klickes *et al*., 1996). This may explain the predominance of MAT α strains among environmental and clinical isolates. Differences in biology, virulence, clinical features, epidiomology, drug susceptibilities have been reported to be related to mating types varieties species and molecular types within the *Cryptococcus* species complex (Kwon-Chung *et al*., 1992; Speed and Dunt, 1995; Casadevall and Perfect, 1998; Meyer *et al.,* 2003; Tritles *et al*., 2004; Campbell *et al.,* 2005; Fraser *et al*., 2005). Strains of serotype A MAT *C. neoformans* var. neoformans commonly found in Europe has been reported to be less virulent and more susceptible than *C. neoformans* var. grubii to fluconazole (Fortorano *et al*., 1997; Casadevall and Perfect, 1998). Virulence factors are those mechanisms that enable the fungus to inflict damage to host (Casadevall and Pirofski, 1999, 2003). Mating types and serotypes have both been implicated as virulence factors in *C. neoformans* (Xlin *et al*., 2008). From existing knowledge, mating types has only been detected using the PCR technique. Since antifungal susceptibility tests has been used in identifying serotypes, here in this work, attempt has been made to identifying mating types, using the reaction of strain to fluconazole. Mating tests are carried out using the method described by Kwon-Chung and Beneth (1978). In this work, two strains have been selected to verify this claim.

MATERIALS AND METHODS

Strain Preparation: Nine clinical isolates were obtained from blood samples which had been

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collected, tested and discarded in the University of Benin Teaching Hospital without having contact with patients. Six environmental isolates were obtained from pigeon droppings in Benin City. They were grown on sabouraud Dextrose Agar (SDA) and the viabilibitywas maintain on slant in +4°C.

*Confirmation of Isolates via Selective Medium-Niger Seed Agar and Thermotolerance***:** Niger seed medium was used as selective medium for the confirmation of C. neoformans in this work.The isolates were confirmation by the production of melanin (dark pigmentation) (Lazera *et al.,* 1997) after incubation for at least 48 hours, at a temperature of 37°C. The ability to grow at this temperature range was also used as a mark for the selection.

Antifungal Susceptibility Testing: Environmental isolate NBM 5 and clinical isolate 4832 were selected for testing with fluconazole because of the conspicuous production of melanin on Niger seed Agar suggesting high virulence. Antifungal susceptibility testing was carried out according to the Clinical and Laboratory Standard Institute (CLSI) document on both microdilution method (CLSI, 2002). A stock solution of 150mg fluconazole was prepared freshly at each testing to a concentration of 7.5mg/ml. Final fluconazole concentrations ranged from 16 – 64mg/ml. the various concentrates were added to the medium inoculated with 100µl of isolates in various dilutions.

Minimum inhibitory concentrations were determined visually by the concentration that inhibited more than 50% of growth compared with the positive control.

Statistical analysis: SPSSV 9.5 was used for the statistical ananlysis. The differences in the means of response of the treatments (clinical and environmental isolates) to Fluconazole was analysed using one-way Anova confirmed with Duncan multiple range test.

RESULTS AND DISCUSSION

Two isolates of *C. neoformans* one clinical (CD 4832), the other environmental (NBM5) were selected for antifungal susceptibility test of which Fluconazole was used. Antifungal efficacy of Fluconazole and its broad spectrum activity has been proved. It is known that Fluconazole is an active agent against wide variety of yeasts and filamentous fungi(Sckhon, Garg and Hamit, 1990; Mallic *et al.,* 1990) These isolates were selected based on their growth and melanin production on a selective medium – Niger seed agar and their thermotolerant ability; growth at 37°C.

The results of antifungal susceptibility test showed that the minimum inhibitory concentration of fluconazole on *Cryptococcus neoformans* is 16mg/ml (Hagen *et al*., 2012). From table 1and 2 it was clear that that environmental isolates is more susceptible to fluconazole than the clinical samples. This is understandable, since clinical isolates may be more virulence due to their active involvement in cases of Cryptococcosis. In attempting to find out if these isolates behaved differently as mating types, they were mixed together in equal concentration. The mixed isolates were incubated on the same medium in the presence of fluconazole. For a very clear result, the highest concentration 64mg/ml used in Table 1 and 2 was maintained for the mixed culture. The results clearly demonstrated that the mixed culture has developed more resistance to the antifungal drug – fluconazole. This was reflected generally in the increase in the number of colonies in the diluted inoculum (10^{-9}) concentration. The result showed that the two isolates behave differently – this was reflected in the change of reaction to fluconazole.

Cryptococcus neoformans isolated from blood samples is expected to behave differently from that of the environmental samples. Based on this assumption, the two isolates were involved in a mixed culture in order to certify their ability to recombine in sexual recombination. This result suggested the term, reproductive advantage for *C. neoformans*. Here in this research, this term is explained as the result of sexual recombination (Table 3) that led to the development of more resistance to a certain antibiotic. This resistance was not attainable in the presence of the individual isolates. The reproductive advantage is bias toward the environmental isolate that was previously highly susceptible to fluconazole. However, the higher level of resistance due to reproductive advantage is only attainable upon the mixing up of the two strains of *C. neoformans*. This shows that pathogenic organisms have the capacity to develop resistance to antibiotic through sexual recombination. According to Lanirs and Idnum (2015), pathogenic microbes are difficult to handle due to the ease with which they develop resistance to the existing drugs. Sexual recombination may be the means by which this resistance is attained. This suggests that the genetic system of Cryptococcus must be highlighted for the understanding of the pathogenicity (Nielsen *et al*., 2005).

Antifungal drug treatment based on azoles, flucytosine and amphotericin B can be effective in treating cases caused by Cryptococcus (Leeh, Chang and Kwon-Chung, 2010). However, prolonged use of these drugs as lifelong prophylactics can lead to drug resistance.

Table 1: Response of clinical isolate to Fluconazole: Colony count per plate of Strain CD4832

Concentration of Fluconazole	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
64µg/ml	2016	648	312	272	240	216	168	128	32
32µg/ml	2120	960	496	192	264	232	200	152	104
16µg/ml	1536	664	280	176	112	80	56	120	48

Table 2: Response of environmental isolate to Fluconazole: Colony count per plate of Strain NBM 5

Concentration of Fluconazole	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
64µg/ml	1004	60	30	13	5	2	4	4	4
32µg/ml	320	18	3	12	10	0	0	0	0
16µg/ml	580	26	23	11	2	2	1	0	0

Table 3: Response of both clinical and environmental isolates: Colony count per plate of combination of strain 4832 x NBM 5

Concentration of Fluconazole	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
64µg/ml	1496	780	640	408	506	600	576	504	384

Table 4a: Analysis of response of treatments to antibiotics (Fluconazole)

Response to antibiotics				
	df	Mean Square	F	Sig.
Between Groups	2	641587.111	3.219	.058*
Within Groups	24	199282.324		
Total	26			

**Significant> 0.05*

Table 4b: Analysis of response of treatments to antibiotics (Fluconazole)

Duncan			
		Subset for alpha = 0.05	
Treatment	N	1	2
environmental	9	125.1111[a]	
clinical	9	448.0000[a]	448.0000[a]
combination	9		654.8889[b]
Sig.		.138	.335

This in the view of (Mondon *et al*. 1999) may be due to the presence of "heteroresistance". In the context of this work, heteroresistance may be involved when two strains from clinical and environmental sources are mixed together and allowed to incubate in the same medium. This makes identification and development of new drug targets for treatment of Cryptococcus an active area of research. This was what necessitated the exposure of the strains to fluconazole. Since the two strains used in this work showed statistical differences (table 4a and 4b) in their susceptibility to fluconazole at the level of recombination, this suggested two mating types.

Interestingly, when the two strains were mixed together a higher level of resistance ensued. This immediately brought to mind the development of heteroresistance to antifungal drug due to different mating types In conclusion, this work has demonstrated that mating types can also be determined using virulence approach apart from PCR methodology. This may be easier in the selection of strains for further research work

REFERENCES

Barchiesi, F; Cogliati, M; Esposto, MC; Spreghini, E ;Schimizzi, AM; Wickes, BL; Scelise, G; Viviani, MA (2005). Comparative analysis of pathogenicity of *Cryptococcus neoformans* serotypes A, D and AD in immune Cryptococcosis. *J. Infect*. 51: 10 – 16.

Boekhart, T; Theilen, B; Diaz, M; Fell, J; Hop, W.C; Abeln, EC *et al*. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* 147: 891 -907.

Boekhont, T; Van Belkum, A; Leenders, AC; Verburgh, HA; Mukamurangwa, P; Swinne, D; *et al*. (1997). Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. *Int. J. Sys. Bacteriol*. 47: 432 – 442.

Byrnes, EJ; Li, H; Lewit, Y; Ma, H; Voelz, K; Ken, P *et al*. (2010). Emergence and pathogenicity of highly virulent *Cryptococcus gatti* genotypes in

the northwest United States. *PLOS Pathog*. 6: e1000850.

Campbell, LT; Fraser, JA; Nichols, CB; Detrich, FS; Carter, P; Heitman, J (2005). Clinical and environmental isolates of *Cryptococcus gattis* from Australia fecundity. *Gykaryot Cell* **4:** 1410 – 1419.

Casadevall, A and Pinifski, LA (1999). Host-pathogen interactions redefining the basic concepts of virulence and pathogenicity. *Infect. Immun.* **67**:3703 – 3713.

Casadevall, A; Perfect, R (1998). *Cryptococcus neoformans*. Washington ASM Press.

Casadevall, A; Pirofski, LA (2003). Microbial virulence results from the interaction between host and microorganism. *Trends Microbiol*. **11**:157 – 158

Chau, T.T Mai, NH; Phil, NH; Nghia, HD; Chuong, LV; Sinh, DK *et al*. (2010). A prospective descriptive study of Cryptococcal meningitis in HIV uninfected patients in Vietnam – high prevalence of *Cryptococcus neoformans* var. grubii in the absence of underlying disease. *BMC Infect. Dis.* **10**: 199.

Del Poeta, M and Casadevall, A (2012). Ten challenges on Cryptococcus and Cryptococcosis. *Mycopathologia* 173: 303 – 10.

Fraser, J; Gites, S; Wenrok, E; Geunes-Boyer, SG; Wright, JR; Diezmann, S; Allen, A; Stajich, JE; Dietrich, FS; Perfect, IR *et al*. (2005). *Cryptococcus gatti* outbreak. *Nature* 437: 1360 – 1364.

Hagen, F; Colom, MF, Swinne, D; Tintelnot, K; Latta, R; Montagna, MT *et al*. (2012). Autochthonous and dormant *Cryptococcus gatti* infections in Europe. *Emerg. Infect. Dis.* 18, 1618 – 1624.

Hagen, F; lllnait-Zaragozi, M; Meis, JF; Chew, WHM; Curfs-Breuker, I; Mouton, JW; Hoepelman, AIM; Spanjaard, L; Verweij, PE; Kampingaf, GA; Kuijper, EJ; Boekhout, T and Klaassen, CHW. (2012). Extensive genetic diversity within the Dutch clinical *Cryptococcus neoformans* population, *Journal of Clinical Microbiology* **50**(6): 1918 – 1926.

Halliday, CL; Bui, T; Krockenberger, M; Malik, R; Ellis, DH and Carter, DA (1999). Presence of α and a mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. gatti strains from Australia. *J. Clin. Microbiol.* **37**:2920 – 6.

Kessler, AT; Alkharrat, T; Kourtis, AP (2010). Cryptococcus neoformans as a cause of bronchiolitis obliterans organizing pneumonia. *J. Infect. Chemother*. 16, 206 – 209.

Kidd, S.E; Hagen, F; Tscharke, RL; Huynb, M; Bartlett, KH; Fyfe, M *et al*. (2004). A rare genotype of *Cryptococcus gatti* caused the cryptococcosis outbreak on Vancouver Island (British Colombia, Canada). *Proc. Nali. Acad. Sci.* **101**: 17258 – 172263.

Kwon-Chung, KJ (1996). Morphogenesis of *Fitobasidiella neoformans*, the sexual state of *Cryptooccus neoformans*. *Mycologia 68*: 942 – 946.

Kwon-Chung, KJ Edman, JC; Wickes, BL (1992). Genetic association of mating types and varieties in *Cryptococcus neoformans*. Infect. Immun. 60: 602 – 5.

Kwon-Chung, KJ; Bennett, JE (1978). Distribution of α and a mating types of *Cryptococccus neoformans* among natural and clinical isolates. *Am. J. Epidemiol.* **108**: 337 – 341.

Kwon-Chung, KJ; Varmaff, D (2006). Major species concept support one, two or more species within *Cryptococcus neoformans*? *FEMS yeast Res*. **6**: 574 – 87.

Lazira, MS; Cavalcanti, MAS; Triles, L; Nishikawa, MM; Nante, B (1998). *Cryptococcus neoformans* var. gatti – evidence for a natural habitat relating to decaying wood in a pottery tree hollow. *Med. Mycol.* **36**: 119 – 122.

Leeh SE; Chang Y C; Kwon-Chung K. (2010) Cryptococcus neoformans overcome stress of azole drugs by formation of disomy in specific multiple chromosomes. PloS Pathog 6:e1000848.http://dx.doi.org/10.137/journal.ppat. 1000848

Lin, X; Libvintseva, AP; Nielsen, K; Patel, S; Floyd, A; Mitchell, AG ; Heitman, J. (2007). αADα hybrids of *Cryptococcus neoformans* evidence of same sex mating in nature and hybrid fitness. PLOS Genet, 3: e1861990.

Lin, X; Niesen, K; Patel, S and Heitman, J (2008). Impact of mating type, serotype and Ploidy on the virulence of *Cryptococcus neoformans*. *J. Infect. Immun.* 2923 – 2938.

Litrintsewa, AP; Kestenbaun, L; Vilgalys, R and Mitchell, TG (2005). Comparative analysis of environmental and clinical populations of *Cryptococcus neoformans. J. Clin. Microbiol.* 43: 556 – 564.

Madinez, LR and Casadeval, A (2006). Susceptibility of *Cryptococcus neoformans* biofilms to antifungal agents in vitro. *Antimicrob. Agents Chemother*. 50, 1021 – 1033.

Mallic M, Montes B, Royne S J; Bastide J M, (1990) Susceptibility of various species of Candida and Torulopsis to Fluconazole and Ketoconazole. *Drug Exp. Clin.Res*. **14**: 507-513

Meyer, H; Castaneda, A; Jackson, S; Huynh, M; Castaneda, E. (2003). Mo-leader typing of Ibero American *Cryptococcus neoformans* isolates. *Emerg. Infect. Dis.* 9:189 – 195.

Mondon P; Petter R; Amalfitano G; Luzzati R; Concia E; Polacheck I; Kwon-Chung K, (1999). Heteroresistance to Fluconazole and voriconazole ic Cryptococcus neoformans. *Antimicrob. Agents-Chemother* **43**: 1856-1861

National Committee for Clinical Laboratory Standards (2002). Reference method for birth dilution antifungal susceptibility testing for yeasts approved standard; 3rd ed; Document M27 – A2. National Committee for Laboratory Standards, Wayne, PA.

Negroni, R (2012). Cryptococoosis. *Clin. Dermatol.* 30, 599 – 609.

Nielsen K; Marra R F; Hagen F; Boekhout T; Mitchell T G; Cox, GM; Heitman J (2005). Interaction between Genetic Background and Mating-Type Locus in *Cryptococcus neoformans* Virulence Potential DOI: 10.15 34/genetics.105.045039

Park, BI Wannemuehler, KA; Marston, BJ; Govender, N; Pappes, PG; Chiller, TM (2009). Estimation of the current global burden of Cryptococcus meningitis among persons living with HIV/AIDS. *AIDS*, 23: 525 – 30.

Sckhon AS; Garg A K; Hamiz Z (1990) Effect of culture media on invitro susceptibility of fluconazole against some yeasts. *Mycoses* **34**: 319-322.

Speed, B; Dunt, D (1995). Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans. Clin. Infect. Dis.* 21: 28 – 34;

Tortorans, AM *et al*. (1997). Prevalence of serotype D in *Cryptococcus neoformans* isolates from HIV positive and HIV negative patients in Italy. *Mycoses* 40: 297 – 302.

Trilles, L; Warg, B; Fircacative, C; Lazera, MS; Klarke, B; Meyer, W (2014). Identification of the major molecular types of *Cryptococcus neoformans* and *C. gatti* by hyperbranched rolling circle amplification. *PLOS One,* 9: e94648.

Van, Z; Li, X; Xu, J (2002). Geographic distribution of mating type alleles of *Cryptococcus neoformans* in four areas of the United States. *J. Clin. Microbiol.,* 40: 956 – 972.

Wickes, BL; Mayorga, ME; Edman, U; Edman, JC (1996). Dimorphism and haploid fruiting in Cryptococcus neoformans association with the alpha-mating type. *Proc. Nat. Acad. Sci.* **93**: 7327 – 7331.