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Development of Congenic Mutants and Genetic Analysis of Virulence in Cryptococcus Neoformans

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ABSTRACT: *Cryptococcus neoformans* strains were isolated from clinical source taken from the University of Benin Teaching Hospital (UBTH) and environmental source taken from New Benin Market. Two strains were selected; clinical isolate 4832 and environmental isolate NBM5. They were determined to be of two different mating types, due to differences in their susceptibility to the antifungal agent fluconazole. In order to check the involvement of mating type locus in the virulence of *C. neoformans*, congenic mutants were developed. Based on genetic analysis, the mating type locus in synergy with other loci influence the virulence in *C. neoformans.* Genetic recombination in this organism unfolds the reality of horizontal gene transfer in *C. neoformans* which could also apply to other organisms. In conclusion, most resistance of microbial organism to antibiotics could be as a result of the genetic recombination following selection.

Keywords: Congenic mutants, mating type locus, virulence, genetic recombination and horizontal gene transfer.

Introduction

Cryptococcus neoformans is a basidiomycetous yeast that is pathogenic and the cause of cryptococcal meningitis in humans. The spores of the fungus are inhaled from the environment and both immunocompromised and immunocompetent individuals are susceptible. Cryptococcal meningitis can be deadly and is the cause of more than half a million deaths annually around the world (Park *et al.,* 2009). The propagation of *Cryptococcus neoformans* is majorly clonal though evidence of mating type machinery, mating type loci and either a parasexual or sexual life cycle have been provided (Litvintseva *et al.,* 2003). Also, substantial evidence that mating types are associated with virulence has been provided (Davidson *et al.,* 2003). *C. neoformans* has two mating types α and a. The former is known to be prevalent and a cause of most cryptococcal infections.

The *C. neoformans* species complex is a monophyletic lineage which involves two species; *C. neoformans* and *C. gattii. C. neoformans* is the cause of most cryptococcal infections within the species being classified into varieties; var. *grubii* (serotype A), var. *neoformans* (serotype D) and the hybrid serotype AD (Casadevall and Perfect, 1998; Kwon-Chung and Varma, 2006; Lin and Heitman, 2006). The effect of mating types on virulence of *C. neoformans* seems to be strain dependent (Heitman *et al.,* 1999; Nielsen *et al.,* 2005). Congenic strains are a favoured method for the study of cryptococcal virulence and morphogenesis (Bing *et al.,* 2013).

Congenic pairs are strains that are genetically identical except at their mating type loci (MAT) (Zhai *et al.,* 2013). Congenic pairs can be constructed by a series of ten backcrosses. The virulence of these strains differs from their parental strains and the genetic background of the congenic pairs can influence the impact of the mating type allele on virulence (Nielsen *et al.,* 2005). The *C. neoformans* congenic strains JEC21α and JEC20a were the first to be constructed and were widely used, constructed in the 1990s and then sequenced in the early 2000s (Kwon-Chung and Varma, 2006; Loftus *et al.,* 2005). They were developed from their progenitor serotype D strains, NIH433(a) and NIH12(α) (Kwon-Chung and Varma, 2006, Heitman *et al.,* 1999). The congenic pairs KN3501α/KN3501a and KN433α/KN433a are also related serotype D congenic strains that were constructed for *C. gattii,* AIR265α and AIR265a (Zhu *et al.,* 2013).

In this work attempt has been made to underpin the direct involvement of the mating type locus in the virulence of the *C. neoformans* strains using constructed congenic strains which aid the genetic analysis. The mating type locus of *C. neoformans* spans ~105 to 130kb and contains >20 genes (Lengeler *et al.*¸2002).

Materials and methods

Strains preparation: The two strains of *C. neoformans* used in the work were selected from the previous work done in the Department of Plant Biology and Biotechnology. The environmental isolate (NBM5) was obtained from pigeon droppings while the clinical isolate (4832) from blood samples which had been collected, tested and discarded in the University of Benin Teaching, Hospital Benin City, without having contacts with the patient. Both strains were routinely cultured on Sabouraud dextrose agar medium or Yeast Peptone Dextrose agar (YPDA) following standard laboratory procedures. Isolates were maintained on fresh broth with sub-culturing every two-three weeks. Isolates were regularly cultured at 37 °C

Reaction of clinical and environmental strains to fluconazole prior to the development of congenic mutants: Based on the minimum inhibitory concentration already established according to Eboigbe and Usiosefe (2019), both strains by serial dilution were tested with 16 ug/ml fluconazole in YPDA plates.Their susceptibility to fluconazole was observed. This test was carried out in accordance with the Clinical and Laboratory Standard Institute (CLSI) document on both microdilution method.

Development of congenic mutants: Congenic mutants for isolates NBM5 and 4832 were generated according to Nielsen *et al*. (2005) model. The isolates NBM 5 and 4832 were inoculated on Sabouraud dextrose agar for 24 h at 37 °C to obtain a single spore. The single spore was used in generating the congenic mutants. The parent strains NBM5 and 4832 were crossed in broth media from single spores and incubated at 37 °C for 24 h. The broth culture was then poured on Sabouraud dextrose agar plate and incubated at 37 °C for 24 h to generate a single spore from the cross. One of the F1 progeny was then backcrossed separately to single spore of NBM5 and 4832 isolates respectively. This process of backcrossing of the subsequent progeny to the original parents was repeated nine times, in order to generate congenic mutants at the end of the tenth generation.

Reaction of congenic mutants to fluconazole: After the development of the congenic mutants as stated above, the strains were again challenged with the same concentration of fluconazole (16 ug/ml) following the same procedure stated above. This step was necessary in order to compare the reaction of the clinical and environmental strains with the congenic mutant strains thus developed.

Virulence analysis of congenic mutants: Ten (10) mice were challenged with 1:1 ratio of the two congenic mutants (1.00 X 10^6 total fungal cells per animal) intravenously. Five mice were for each congenic mutant. The numbers of inocula for the congenic mutants were estimated by measuring the colony forming unit (CFU) of serial dilution. Two mice without inoculation were used as positive control. The mice were left after post-inoculation for forty (40) days.

Results

From our previous work on the screening and selection for *C. neoformans*, we noted two strains for their melanin production. Strain4832 were identified from clinical source and NBM5 were isolated from environmental source. Both strains were first tested for their reaction to fluconazole at 16µg/ml. The result of their independent reactions reflected a clear difference between the strains (Table1). Thus, the strains were regarded as different mating types. This difference has been associated with the mating type locus (Eboigbe and Usiosefe, 2019)

Table 1: Response of environmental and clinical isolates to Fluconazole: Colony count per plate

Concentration of Fluconazole(16 µg/ml)	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
Environmental sample (NBM5)	580	26	23	11	02	02	01	00	00
Clinical sample(4832)	1536	664	280	176	112	80	56	120	48

Following their differences (in the reaction) to fluconazole, the decision to develop congenic mutants became obvious. Firstly, congenic strains were developed in order to obtain strains that differed only at their mating type locus. The congenic strains were developed from strains 4832 and NBM5 by crossing both parent strains

through a series of backcrossing nine times. As a result of the repeated backcrossing, genetic recombination would take place such that the strains are the same except at their mating type locus. Since they differ from each other only at their mating type locus, the first cross that were used to generate the congenic mutants were thus labeled as B4532α and B4532a respectively (Fig.1).

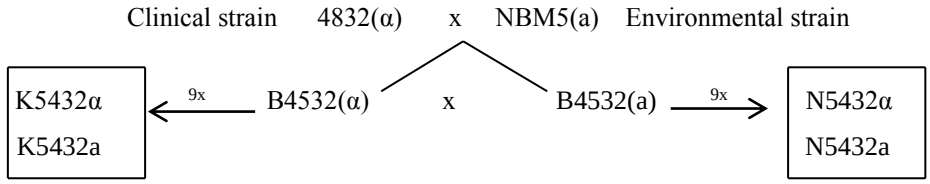

Figure 1: Crossing Strategy for generating Congenic mutants**.** B4532α and B5432a were generated from the cross between 4832α and NBM5a. K5432α/K5432a was generated from repeated backcrossing using B4532α and N5432α/N5432a was generated from repeated backcrossing using B4532a

In an attempt to analyze their genetics recombination, two strategies were employed, firstly the congenic mutants were exposed to fluconazole, and secondly the congenic mutants were used to challenge a set of mice of the same sex. The results showed that, genetic recombination took place during the development of the congenic strains (Table 2).

Table 2: Response of congenic mutants to Fluconazole:Colony count per plate

Concentration of Fluconazole(16 µg/ml)	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
Congenic mutant1 (B5432a)	>8000	6018	341	210	177	151	97	49	68
Congenic mutant2 (B5432α)	527	162	34	04	07	01	02	04	04

Unfortunately the results of the reaction of the mice to the congenic mutants did not reflect the same tone as that of fluconazole. Forty days after post inoculation, all the mice injected with the congenic mutants remain alive indicating no differences in the reaction.

Discussion

In an attempt to analyze the impact of mating type locus on the virulence of *C. neoformans*, a cross between clinical isolate which was regarded as the α-mating type and environmental isolate typifying the a-mating type was carried out. The difference in their mating types was determined based on their reaction to fluconazole, as it is commonly accepted that the α-mating type are usually more virulence (Kwon-Chung, 1981). From the genome sequences of *C. neoformans* available online, the sequences of congenic mutants with 'α' and 'a' mating type loci were isolated. Comparing the two sequences using the NCBI blast for alignment, it was interesting to note that the only difference between their sequences was at the mating type locus (Kruzel, Giles and Hull, 2012). Under this backdrop, we decided to develop a congenic mutant between the selected *C. neoformans* strains (4832 and NBM5). According to Zhai *et al*.,(2013), congenic mutants are strains that are genetically identical, except for the mating type locus (mat)

Based on the above facts and following a well-established protocol, congenic mutant pairs (Fig.1) were developed. Accordingly, we attempted to use the congenic mutant thus constructed as a genetic marker in examining and understanding the genetics of *C. neoformans* and consequently its role in the virulence. In order to implicate the involvement of the mating type locus in virulence, mice were injected intraveinously with congenic mutants as constructed in (Fig.1). However, there was no difference in reaction to the congenic mutants and no mortality in any of the mice, thus suggesting that the mating type locus is not solely involved in the virulence of *C. neoformans*. We thus judge since from our previous analysis of the parental stock the clinical isolate (4832) was more virulent to the environmental isolate (NMB5). Other loci in accordance with Kronstad, Hu and Choi (2011) may play a more direct role in virulence. Probably, the mating type loci are acting in synergy with other loci in contributing to virulence. A study of this nature is important since the genes (in the mating type locus) and other linked loci that have an overall impact on virulence will enable the discovery of novel targets for drugs in combating cryptococcosis.

In order to further examine the congenic mutants developed, we decided to re-evaluate their reaction to fluconazole. Interestingly, there was a clear difference between the congenic pairs of which that from the environmental origin became more virulent (table2). This reverse trend compared with the previous reaction

(table1) is a clear evidence of genetic recombination between the two strains used. For the fact that the mutant from the environmental origin became more virulent contrarily to expectation, may suggest a case of "horizontal gene transfer" in *C. neoformans* strains. Furthermore, this is a case of neutral acquisition (as in mice response) which probably later provide novel recombination of genetic material for selection to act on (Soucy, Huang Gogarten, 2015). Zhai *et al*. (2013) also suggested that genes present in the mating type locus may be of biological importance and give an idea of how *Cryptococcus neoformans* is able to adapt to new conditions and acquire resistance to presently available antifungal drugs.

In conclusion, the congenic mutants thus developed have revealed in this work, the possibilities in recombination of genetics materials in *C. neoformans* which may also apply to other organisms. It is clear that many of the resistance to antibiotic or other agents developed in microbial organisms could be as a result of genetic recombination which is common among organisms that are sexually or vegetative compactible.

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