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Isolation, characterization and pigment production from *Cryptococcus neoformans* in pigeon droppings and their UV susceptibility

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ABSTRACT

C. neoformans was isolated from the pigeon droppings, out of 50 samples 3 isolates of *C. neoformans* was isolated and further studied for melanin pigment production using L-DOPA medium. Both melanised and non-melanised pigment was further subjected to UV susceptibility and the survival rate was studied at 254 nm based on the different old days and different exposure time. In this study the results release that melanised cells are able to survive than non-melanised which states melanin pigment has a capacity to protect itself from the various environmental factors to some extent which was absent in non-melanised.

Key words: Isolation, characterization, *Cryptococcus neoformans*, UV susceptibility.

INTRODUCTION

Cryptococcus neoformans is the etiological agent of Cryptococcosis that has a world wide in distribution. The pathogen occurs as a saprobe in a wide variety of natural substrate including avian excreta, soil, vegetables, fruits and eucalyptus (Emmons *et al.*, 1955). Prevalence of *C. neoformans* in the environment has been mainly associated with soil and pigeon excreta. Thus yeast has been frequently recovered from pigeon droppings is because of its high contents of nitrogen source (Creatinine) which favors the growth of the organism, therefore their excreta is the most important habitat for *Cryptococcus neoformans*. Pigeons do not suffer from the disease presumably, because of their high body temperature (41.5°C-43.3°C).

Polysaccharide capsule is the major virulent factor of C. neoformans. Melanin is second major virulent factor. Synthesis of pigment melanin (light to dark brown) by *C. neoformans* occurs only in the presence of phenolic compounds (L-dopa), 4, 3, L-dihydroxy phenyl alanine and is catalysed by the enzyme phenol oxidase (Laccase). Melanin is deposited in their cell wall and comprises approximately 15% of the dry weight of late stationary phase – melanised *C. neoformans* (Williamson *et al.,* 1997). Three major types of melanin are recognized eumelanin, phaeomelanin, allomelanin. The presence of melanin in *C. neoformans* may also have a role in determining the environmental habitat and infectious origin of this organism since essentially all pathogenic *Cryptococci* are pigmented.

In additional, it is plausible that melanin protects. Cryptococcus in its natural environment by its protective properties against UV irradiation or from the oxidative stress. Melanisaton of *C. neoformans* in the environmental sources increases their survival rate. Further more, in vitro studies shown that non-melanised *C. neoformans* are highly susceptible to enzymatic degradation by soil-inhabiting protozoa and bacteria. Recent studies have shown that *C. neoformans* can synthesis compounds in pigeon excreta. The present study aims at to isolate, identify *C. neoformans* from pigeon droppings, demonstration of their pigment production and their UV susceptibility.

MATERIAL AND METHODS

Sample collection

50 samples of pigeon droppings were collected in clean plastic zip-lock bags separately. The samples were collected from three different places in Tamil Nadu (2 places of religious places, pigeon coops at near north Chennai and a place of Guindy National park, Chennai, India). The samples were brought to the laboratory for processing. The samples were kept in a refrigerator until use.

Identification of isolate (Emmons, 1951)

Colony morphology

Gram staining

The isolated colonies were subjected to gram's staining to observe their morphology.

Capsule staining

The negative staining of the isolated colonies was prepared using Nigrosin (Indian Ink) and observed under oil objective.

Biochemical reactions

The presences of biochemicals were evaluated by sugar fermentation test, carbohydrate assimilation test and nitrate reductase test.

Demonstration of melanin pigment production

In this study the environmental isolate of *C. neoformans* identified from pigeon droppings were examined the melanin pigment production in the presence of diphenolic compounds (Chaskes and Tyndall 1978).

Survival of C. neoformans during melanisation and susceptibility to UV light (254 nm) To study relationship between melanisation and susceptibility to UV light, *C. neoformans* culture were grown in the presence and absence of L-DOPA; different age-old cultures were irradiated with UV for 15 minutes at 50 cm distance from the UV lamp (Wang and Casadevall 1994).

Survival of 8 day old melanised and non melanised C. neoformans after irradiation with UV light (254 nm) at different time intervals

To study whether melanised cells were less susceptible to UV light (254 nm) 8 days old melanised and non melanised yeast cells were exposed to UV radiation at different time intervals.

RESULTS

Isolation of C. neoformans from pigeon droppings

Out of fifty samples three isolates of *C. neoformans* were isolated

Macroscopic appearance / Colony morphology

Staib's and Pal's medi

Brown colored colonies were observed on both staib's and pal's medium with in 3-7 days of incubation. Formation of brown clour is due to the oxidative polymerization of the substrate diphenyl compounds into melanin pigment.

Melanin is deposited in their cell wall by producing the enzyme phenol-oxidase (Laccase).

The colonies are highly mucoid 2-3 mm in size.

Tobacco agar

Dark brown colonies were observed

Sabourouds dextrose agar

Colonies are large, smooth, highly mucoid, creamy white in color 2-3 mm in size.

Microscopic appearance

Grams staining

Budding yeast cells ranging from 5-20 µm in size were observed.

Capsule staining

Budding yeast cells ranging from 5-20 µm in size surrounded by a refractive gelatinous capsule.

Biochemical reactions

The results of various biochemicals are listed in table 2

Melanin pigment production

All the isolates of *C. neoformans* were tested for the production of melanin pigment on the defined minimal broth and agar medium with and without L-DOPA. Visual observation of light to dark brown pigment on DMMM agar with out L-DOPA shows white color colonies after 3-7 days incubation at 37°C was indicative of absence of melanin

Table 1: Isolation of *C. neoformans* from pigeon droppings

S. No	Sources of the samples	No of samples	No of isolates recovered	Percentage	No of colonies (CFU/ml)
1	Pigeon coops	20	2	1	34×10^4
2	Religious places	15	-	-	-
3	Guindy National Park	15	1	6.75	6×10^4

pigment. In this defined minimal medium with L-DOPA *C. neoformans* cultures produces brown pigment (melanin) after 3-5 days and heavily melanised after 5-8 days (black).

Glycine was chosen for a nitrogen source this will enhances the production of melanin pigment. L-DOPA (Dihydroxy phenylalanine) acts as a substrate for melanin pigment production.

Survival of *C. neoformans* during melainsation and susceptibility to UV light (254 nm)

At days 2, 3, 6 and 8 old *C. neoformans* cells grown with 1mM L-DOPA (melanised0 were less susceptible to UV light than the cells of the same age grown without L-DOPA (Non melanised). This phenomenon is not the differences in culture grown since addition of L-DOPA does not serve as a carbon or nitrogen source for C. neoformans, therefore it is not produces differences observed between the cells grown with or without L-DOPA are due to the nutritional differences in the medium.

C. neoformans were heavily melanised at 8 day old culture and therefore cells more survival in the medium which were UV irradiated than the cells of 2, 3, 6 and 8 days culture. Where as non-melanised cells of same age culture were more susceptible to UV light.

Table 2: Biochemical reactions of *C. neoformans*

Test	*C. neoformans*
Enzyme production	
Urease	+
Nitrate reduction	-
CHO fermentation test	
Glucose	+
Lactose	-
Sucrose	+
Maltose	+
Galactose	+
CHO assimilation test	
Glucose	+
Lactose	-
Sucrose	+
Maltose	+
Galactose	+

(+) - Positive, (-) - Negative

Survival of 8 days old melanised and non melanised *C. neoformans* after irradiation with UV light (254 nm) at different time intervals

C. neoformans was grown in 1mM L-DOPA became lightly melanised at 3 of culture growth and was heavily melanised by day 8 (naked eye). Non melanisation was observed with *C. neoformans* grown with out 1mM L-DOPA during the course of experiment.

The growth rate of different are old (2, 3, 6 and 8) culture of both melanised and non-melanised results were tabulated. At 8 day old culture of *C. neoformans* with L-DOPA (melanised) were less susceptible to UV than cells of the same age culture grown without L-DOPA (non-melanised).

No cells of non-melanised *C. neoformans* were observed after 20 minutes of UV irradiation but the cells of melanised *C. neoformans* were observed as less susceptible than that of non

Table 3: Survival of different age –old culture of melanised *C. neoformans*

Days	Original CFU/ml	Duplicate CFU/ml	Average CFU/ml	Percentage of survival CFU/ml	Control CFU/ml
2	5.2×10^3	6×10^3	5.6×10^3	28%	2.0×10^4
3	1.1×10^5	1.42×10^5	1.26×10^5	36%	3.5×10^5
6	6×10^5	6.9×10^5	6.45×10^5	43%	1.5×10^6
8	1.2×10^6	1.39×10^6	1.3×10^6	56.5%	2.3×10^6

Table 4: Survival of different age –old culture of nonmelanised *C. neoformans* after irradiation with UV light (254 nm) for 15 minutes

Days	Original CFU/ml	Duplicate CFU/ml	Average CFU/ml	Percentage of survival CFU/ml	Control CFU/ml
2	5.1×10^3	5.3×10^3	5.2×10^3	20%	2.6×10^4
3	5.2×10^4	5.36×10^4	5.36×10^4	14%	3.8×10^5
6	1.3×10^5	1.53×10^5	1.53×10^5	8%	1.8×10^6
8	1.2×10^5	1.24×10^5	1.3×10^5	5%	2.6×10^6

Table 5: Survival of 8 days old melanised *C. neoformans* after irradiation with UV light (254 nm) at different time interval

Time	Original CFU/ml	Duplicate CFU/ml	Average CFU/ml	Percentage of survival CFU/ml
Control	2.25×10^6	2.37×10^6	2.3×10^6	100%
5	1.98×10^6	2.1×10^6	2.04×10^6	88%
10	1.72×10^6	1.82×10^6	1.77×10^6	77%
15	1.32×10^6	1.28×10^6	1.3×10^6	57%
20	8.6×10^5	9.23×10^5	8.9×10^5	39%
25	8.2×10^5	7.6×10^5	7.9×10^5	34%
30	2.5×10^5	2.5×10^5	2.4×10^5	11%

Table 6: Survival of 8 days old non melanised *C. neoformans* after irradiation with UV light (254 nm) at different time interval

Time	Original CFU/ml	Duplicate CFU/ml	Average CFU/ml	Percentage of survival CFU/ml
Control	2.24×10^6	2.96×10^6	2.6×10^6	100%
5	1.20×10^6	1.2×10^6	1.24×10^6	47%
10	5.68×10^5	6.28×10^5	5.98×10^5	23%
15	1.28×10^5	1.32×10^5	1.3×10^5	5%
20	3.64×10^5	4.1×10^4	3.9×10^4	2%
25	-	-	-	-
30	-	-	-	-

melanised cells of the same age culture. Control plates of both the cells (melanised and non melanised) which were non–irradiated with UV light also measured and results were tabulated. Survival rates were determined by counting the number of colonies relative to those on non irradiated control.

DISCUSSION

The most widely used criteria for distinguishing *C. neoformans* from related organisms is by the production of capsulated budding yeast cell and melanin pigment in clinical and environmental isolates. In the present study *C. neoformans* obtained from various environmental isolates showed capsulated yeast cell and produced melanin pigment in the defined minimal media (DMM) with L-hydroxy phenyl alanine (L-DOPA) and occurrence of melanin pigment in the DMM without L-DOPA. Maximum amount of melanin pigment was produced on the 8th day in DMM which contained L-DOPA. These results are in accordance with work of Chaskes and tyndal (1978) that production melanin pigment in DMM media with L-DOPA indicated a wide variation in yield depending on the age of culture, but satisfactory yield of their was obtained on the 8 days old culture. This report was co-relating with present study.

In the present study *C. neoformans* was inoculated in the 2 different DMM one with L-DOPA another one without L-DOPA. The medium which contain L-DOPA the color change was seen from 3rd day and maximum dark brown color was seen on the 8th day. From the 3-8 day the organism, which was grown on the DMM with L-DOPA was plated and observed for the survival of melanised cells when subjected to UV light at 254 nm.

The percentage of survival of the melanised cells was observed more in the 8 days when compared with the 3-5 days old melanised cells. The medium which does not contain L-DOPA produced non melanised cells still the 8th day L-DOPA does not effect the growth of organism, but non melanised are produced due to the absence o L-DOPA. When the non melanised cells are plated and subjected to UV light at 254 nm. The percentage of survival of organism was very less when compared with melanised cells. This results shows that melanised cells protect themselves from the UV light where as non melanised cells are more susceptible to UV light. These results where co relating with the work of Wang and Cesadevall (1994) table 3, 4. *C. neoformans* showed maximum melanin pigment on the 8th day. This melanised cells protects the organism from UV light when subjected to 15 minutes time interval.

When the 8 days old melanised cells was plated and subjected to UV susceptibility at different time intervals from 5 to 30 minutes exposure. The growth rate was maximum when the plates were exposed to 5 minutes, slowly their growth rate decreased when the time intervals was gradually increased by every 5 minutes that is when the melanised plate were exposed to UV light up to 30 minutes. In 5 minutes exposure the growth was found to 88 %, 10 minutes (77%), 15 minutes (27%),

20 minutes (39%), 25 minutes (34%) and 3 minutes (11%). It shows that melanin pigment protects the organism form UV light but when the exposure time as increased gradually the growth rate decreased. This result was co-relating with the work of Wang and Cesadevall (1994).

In case of non melanised 8 days cells when exposed to different time intervals the organism were not able to survive, the growth rate is minimum because non – melanised cells were more susceptibility to UV light. When compared to melanised cells. Non melanised cells were not able to grow when they are exposed to UV light at 254 nm for 25 minutes duration, but melanised cells showed growth rate of about 34%. Thus results reveal that *C. neoformans* melanin pigment has the capacity to protect itself from the various environmental factors to some extent.

REFERENCES

1. Chaskes, S and Tyndal, R.L., Pigment production by *Cryptococcus neoformans* from para and ortho diphenols: Effect of the nitrogen source. *Journal Clinacal Microbiology* **6**: 509-514 (1975).
2. Emmons, C.W., Isolation of *Cryptococcus neoformans* from soil. *Journal of Bacteriology* **62**: 685-690 (1951).
3. Williamson, P.R., Laccase and Melanin in the pathogenesis of *Cryptococcus neoformans*. *Frontiers in Bioscience* **2**: 99-107 (1997).
4. Wang, Y and Casadevall, A., Susceptibility of melanised and non-melanized *Cryptococcus neoformans* to nitrogen and oxygen derived oxidants. *Infect. Immuno,* **62**: 3004-3007 (1994).