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Extracellular Protease and DNase Activities in Clinical and Environmental Isolates of *Cryptococcus neoformans* Species Complex from Central India

Richa Gumasta[1, 2*], Shesh Rao Nawange[1, 2], Shankar Mohan Singh [1, 2], Abhijeet Garg [3] , Ruchi Sethi[1, 2], Ruchika Yadu[1, 2]

1. Department of Biological Sciences, Rani Durgavati University, Jabalpur-482001 (M.P.) India
2. Centre for Medical Mycology, Fungal Disease Diagnostic and Research Center, Society for Research, Diagnosis and Treatment of Human Fungal Diseases, Jabalpur-482002 (M.P.) India.
3. School of Biotechnology, Devi Ahilya Vishwavidyalaya, Indore, (M.P.) India.

ABSTRACT

Enzymes are important not only for the growth and multiplication of the microorganism but also in the infection, penetration of the host tissue and encountering host defense mechanisms. This study aims to investigate extracellular protease and DNase activity in clinical (20) and 120 environmental isolates of *C. neoformans* species complex collected from different localities of central India. DNase test agar containing toluidine blue and Yeast Carbon Base (YCB) agar medium supplemented with 0.1% BSA + 0.01% polypeptone was employed for the screening of DNase and protease production respectively. DNase and protease production was detected by the appearance of clear zones around the colonies. On the basis of enzymatic activity and their Pz values, high protease production (Pz≤0.6) was observed by 14 (11.6 %) environmental and 4 (11.6 %) clinical strains on 5th day, whereas 35 (29.16 %) environmental and 8 (40 %) clinical strains were screened on 8th day of incubation. Similarly 13 (10.83%) environmental and 3 (15 %) clinical strains on the 5th day, however 32 (26.66%) environmental and 8 (40 %) clinical strains on the 8th day of incubation were found to be high DNase producing strains with low Pz value (Pz≤0.6). In the case of protease activity, no significant difference was observed whereas a significant difference has shown by clinical *C. neoformans* and *C. gattii* strains on the 5th day of DNase production ($p < .001$). Extracellular enzymes play a vital role in the pathogenicity and virulence of *C. neoformans* species complex, therefore, enzymes are considered as worthy targets for developing therapeutics.

Keywords: *Cryptococcus neoformans* species complex, Extracellular enzymes, DNase, protease, virulence, Pz value

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***Address for Correspondence:**

Richa Gumasta, Research Scholar, Dept. of Biological Sciences, R. D University, Jabalpur-482001 (M.P.) India

INTRODUCTION:

Opportunistic pathogens *Cryptococcus neoformans* and *Cryptococcus gattii* are the members of *Cryptococcus neoformans* species complex[1]. *C. neoformans* has been classified into two varieties and three serotypes i.e. serotype A for *Cryptococcus. neoformans* var. *grubii,* serotype D for *C. neoformans* var. *neoformans* and serotype AD for the hybrids of serotype A and D. *C. gattii* was previously known as *C. neoformans* var. *gattii* but currently it is considered as phylogenetically related but distinct species and contains serotype B and C[2-5].

Cryptococcus neoformans and *Cryptococcus gattii* both species were reported for the production of a panoply of extracellular enzymes[6] like proteases [7], urease [8-9], phenoloxidase, phospholipases[10] and DNase[11].

C. neoformans infection commonly acquires from the environment [12], the mechanism includes tissue invasion and is disseminated to other organs via the bloodstream from the lung [13-15]. Studies of experimental infection in rats suggested *C. neoformans* produces tissue disrupting substances such as proteolytic enzymes through which it can penetrate the lung parenchyma and histopathological studies suggested *in-vivo* protease production which degrades collagen fibrils in infected tissues [16].

Cryptococcal proteases have been associated with the virulence and are responsible for the destroying host tissues and for digesting several important host proteins such as collagen, elastin, fibrin, fibrinogen, immunoglobulins and complement factors [17, 7, 10].

MATERIAL AND METHODS:

Clinical and environmental isolates:

In the present investigation, environmental and clinical samples were collected from various sources. Environmental sources include decaying woody debris inside the living tree

trunk hollows, fruits, flowers of various plant species, soil, vegetables and desiccated excreta of a few species of caged birds, were collected from different localities of central India[18]. Clinical samples (blood, urine, CSF) of cancer patients were collected from Netaji Subhash Chandra Bose Medical College and Civil Hospital, Jabalpur City (Madhya Pradesh).

Determination of protease production:

Determination of protease production was performed in which all the isolates were evaluated for their ability to secrete protease on solid medium containing Bovine serum albumin (BSA) [19].

Yeast carbon base (YCB) agar medium containing 11.7% YCB (Himedia Mumbai) supplemented with 0.1% BSA plus 0.01% polypeptone, pH was adjusted to 5, sterilized by filtration through a membrane filter of 0.2µm pore size. To prepare 200 ml of medium, 20 ml of the YCB solution will be added to 160 ml of 1.88% melted agar at 56˚C and then 20 ml of the BSA solution will be added to the medium. Twenty ml portions of the media thus prepared were poured into Petri dishes and media were referred to as YCB-BSA. Test isolates were grown on Sabouraud dextrose agar (SDA) for 24hr for 28˚C. Then each isolate was spot inoculated in triplicates and incubated at 37˚C for 5 days. Then the plates were fixed with 10% trichloroacetic acid (TCA) for 2hr and then stained with Coomassie Brilliant Blue G-250. Protease production was detected by the appearance of proteolytic zones around the colonies. Reference strain *C. albicans* MTCC 227/ATCC 10231 served as positive control and strain of *C. glabrata* IHEM 22129 served as negative control.

Determination of DNase production:

The method used for detecting extracellular DNase production by *C.neoformans* sp. The complex was similar to the procedure used for detecting DNase production by *Staphylococci.* Plates of DNase Test Agar (Difco) supplemented with toluidine blue were prepared. Then each isolate was spot inoculated in triplicates and incubated at 30˚C for 7 days. Then the inoculated plates were flooded with 1 N hydrochloric acid. Yeasts that depolymerized DNA contained in the medium exhibited clear zones surrounding the colonies, whereas those organisms which exhibited no extracellular DNase activity showed only the opaque background of precipitated DNA [20, 11].

Measurement and calculation of Pz value

Protease and DNase activity was measured in terms of a ratio of the diameter of the colony to the total diameter of the colony plus zone of precipitation. Scoring was done according to the method described previously[21]. According to different Pz values strains were categorized into 5 different categories: **-**ve (Pz =1) very high Pz group (Negative for enzyme production), **+/-** (Pz = 0.90-0.99) high Pz group, **++** (Pz = 0.80-0.89) Intermediate, **+++** (Pz = 0.70-0.79) low Pz group, **++++** (Pz ≤ 0.69) very low Pz group (large amount of enzyme production).The strains exhibiting (-) and (+) Pz groups were considered negative in the study.

Statistical analysis:

The data analysis was performed using SPSS statistics of independent 't' test and the critical levels of significance of the results were considered at 0.05 levels i.e. P<0.001 was considered significant for DNase activity.

RESULT AND DISCUSSION:

The present study was focused on the determination of DNase and protease activity on the clinical and environmental strains of *Cryptococcus neoformans* and *Cryptococcus gattii.* On the basis of enzymatic activity and their Pz values, clinical and environmental strains has been divided into five ranges, class and Pz groups. Results are shown in (Table 1 & 2).

Table 1. Scoring of Protease and DNase producing environmental isolates of *Cryptococcus neoformans* species complex into 5 different categories according to their different Pz values calculated on the 5th and 8th day of incubation.

Range	Class	Pz Group	Production	Frequency of Environmental isolates			
				5th day		8th day	
				Protease	DNase	Protease	DNase
Pz=1	**-**	Very high	No production	21 (17.5 %)	23 (19.16 %)	10 (8.33 %)	11 (9.16%)
Pz= 0.9. – 0.99	**+/-**	High	Very low	28 (23.3 %)	37 (30.83 %)	11 (9.16 %)	16 (13.33 %)
Pz= 0.8. – 0.89	**++**	Intermediate	Low	27 (22.5 %)	28 (23.3 %)	26 (21.66 %)	27 (22.5 %)
Pz= 0.70 – 0.79	**+++**	Low	High	30 (25%)	19 (15.8 %)	38 (31.66 %)	34 (28.33 %)
Pz= ≤0.69	**++++**	Very low	Very high	14 (11.6 %)	13 (10.83%)	35 (29.16 %)	32 (26.66%)

Table 2. Scoring of Protease and DNase producing clinical isolates of *Cryptococcus neoformans* species complex into 5 different categories according to their different Pz values calculated on the 5th and 8th day of incubation.

Range	Class	Pz Group	Production	Frequency of Clinical Isolates			
				5th day		5th day	
				Protease	DNase	Protease	DNase
Pz=1	**-**	Very high	No production	3 (15 %)	3 (15 %)	1 (5 %)	2 (10 %)
Pz= 0.9. – 0.99	**+/-**	High	Very low	3 (15 %)	5 (25 %)	1 (5 %)	2 (10 %)
Pz= 0.8. – 0.89	**++**	Intermediate	Low	7 (35 %)	3 (15 %)	3 (15 %)	5 (25 %)
Pz= 0.70 – 0.79	**+++**	Low	High	3 (15 %)	6 (30%)	7 (35 %)	3 (15 %)
Pz= ≤0.69	**++++**	Very low	Very high	4 (11.6 %)	3 (15 %)	8 (40 %)	8 (40 %)

Table 3 shows statistical results of enzymatic activity on 85 *C. neoformans* and 35 *C. gattii* isolates of environmental origin, 18 *C. neoformans* and 35 *C. gattii* isolates from clinical samples of a cancer patient using SPSS software.

Table 3. Test of Significance in Proteinase and DNase activities of environmental & clinical *C. neoformans* and *C. gattii* isolates at 5 and 8 days of incubation using independent't' test.

Strain and types	Number of isolates	Days of incubation	Enzymes tested	Positive Production (%age)	Significance
Environmental					
C. neoformans	85/120	5-days		99/120 (82.5 %)	NS
C. gattii	35/120	8-days		110/120 (91.66 %)	NS
Clinical			Protease		
C. neoformans	18/20	5-days		17/20 (85 %)	NS
C. gattii	2/20	8-days		19/20 (95 %)	NS
Environmental					
C. neoformans	85/120	5-days		97/120 (80.33 %)	NS
C. gattii	35/120	8-days		109/120 (90.83 %)	NS
Clinical			DNase		
C. neoformans	18/20	5-days		20/20 (100 %)	S (**p<0.001**)
C. gattii	2/20	8-days		18/20 (90 %)	NS

NS (nonsignificant), S (significant, **p<0.001**)

Protease and DNase activity by the formation of clear zones around the *Cryptococcus* colonies were observed on the 5th and 8th day of incubation.

Figure 1 Protease activity seen by the formation of proteolytic zones around the *Cryptococcus* colonies with low Pz group (Pz=0.48).

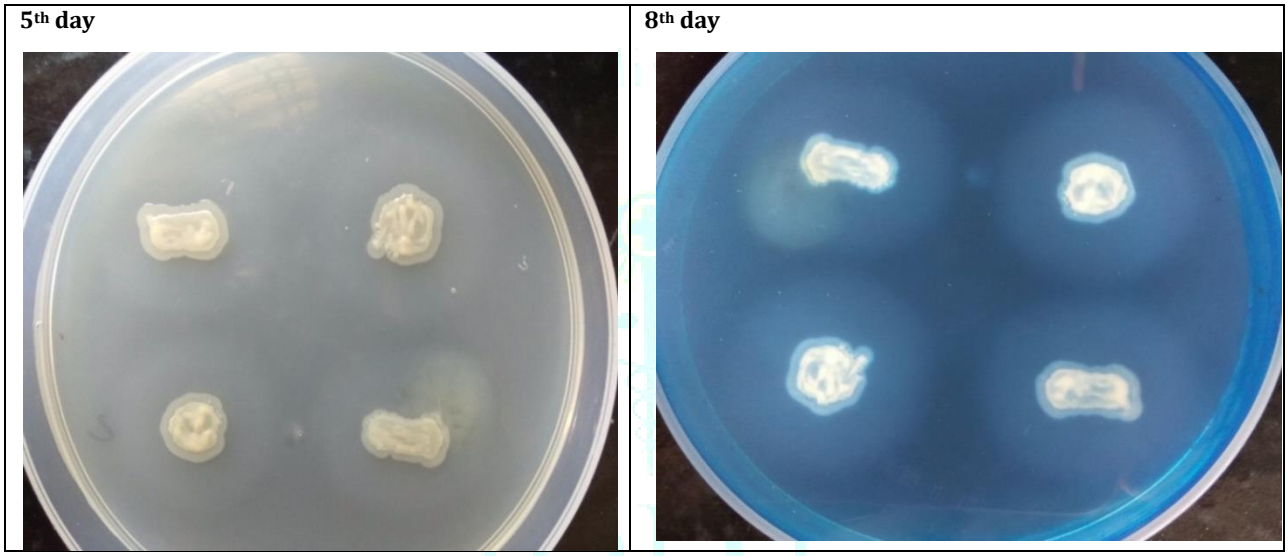
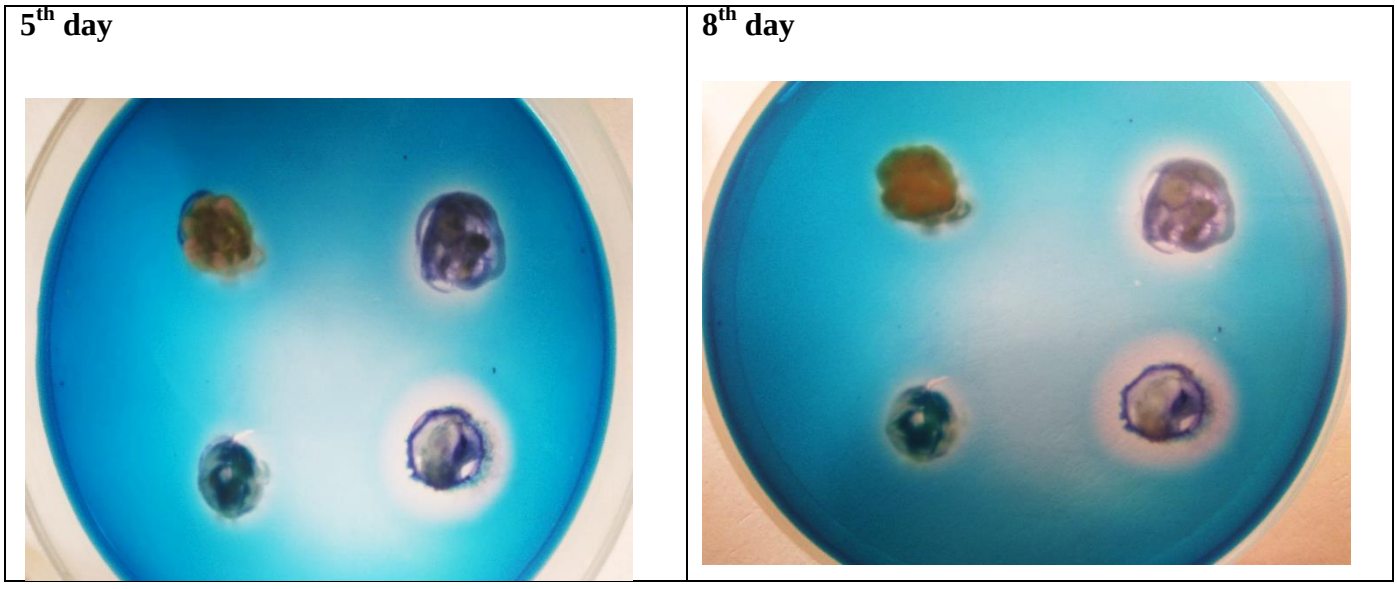
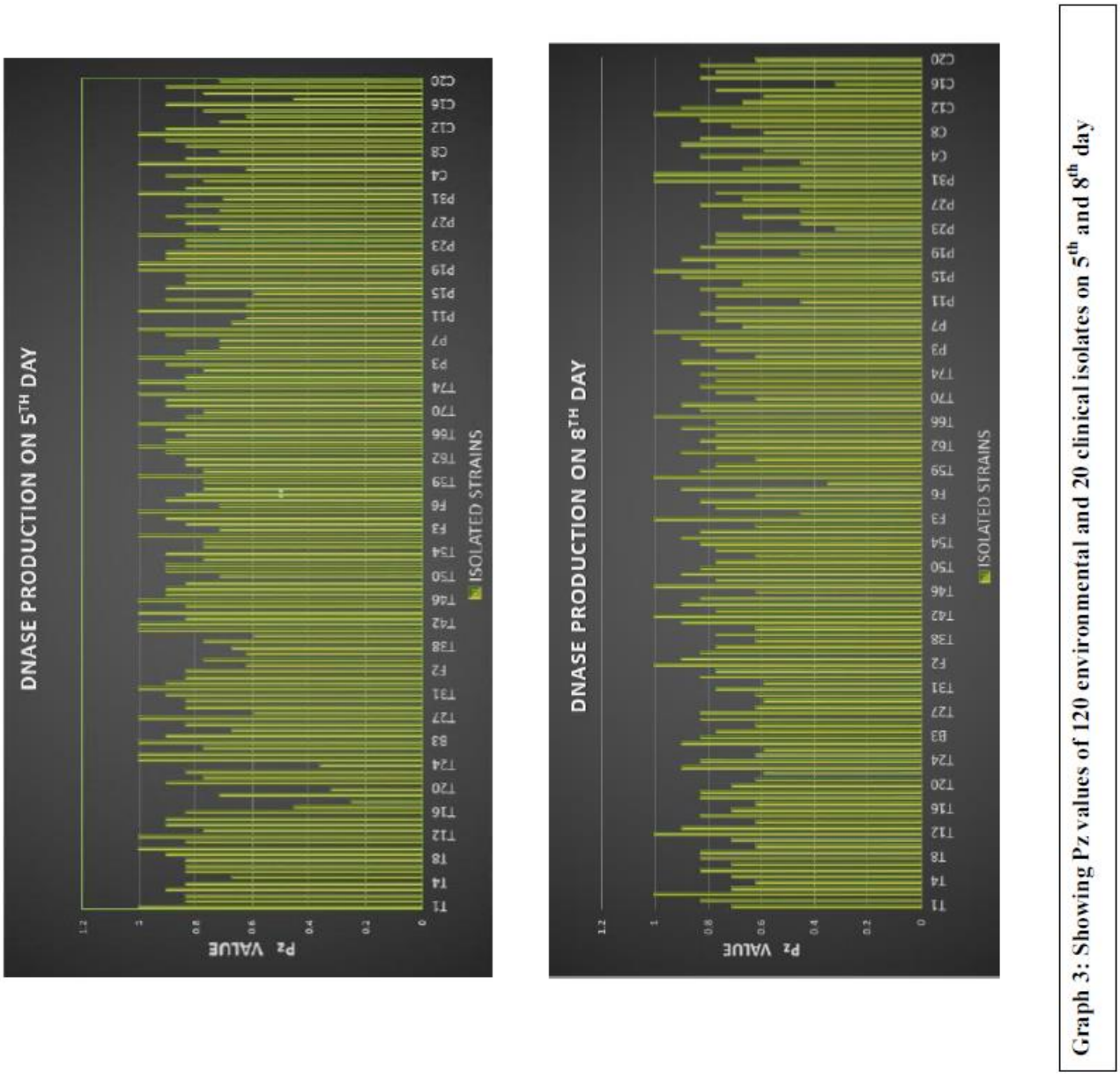


Figure 2. screening results for DNase production by the formation of clear zones around the *Cryptococcus* colonies on the 5th and 8th day of incubation with low Pz group (Pz=0.51)

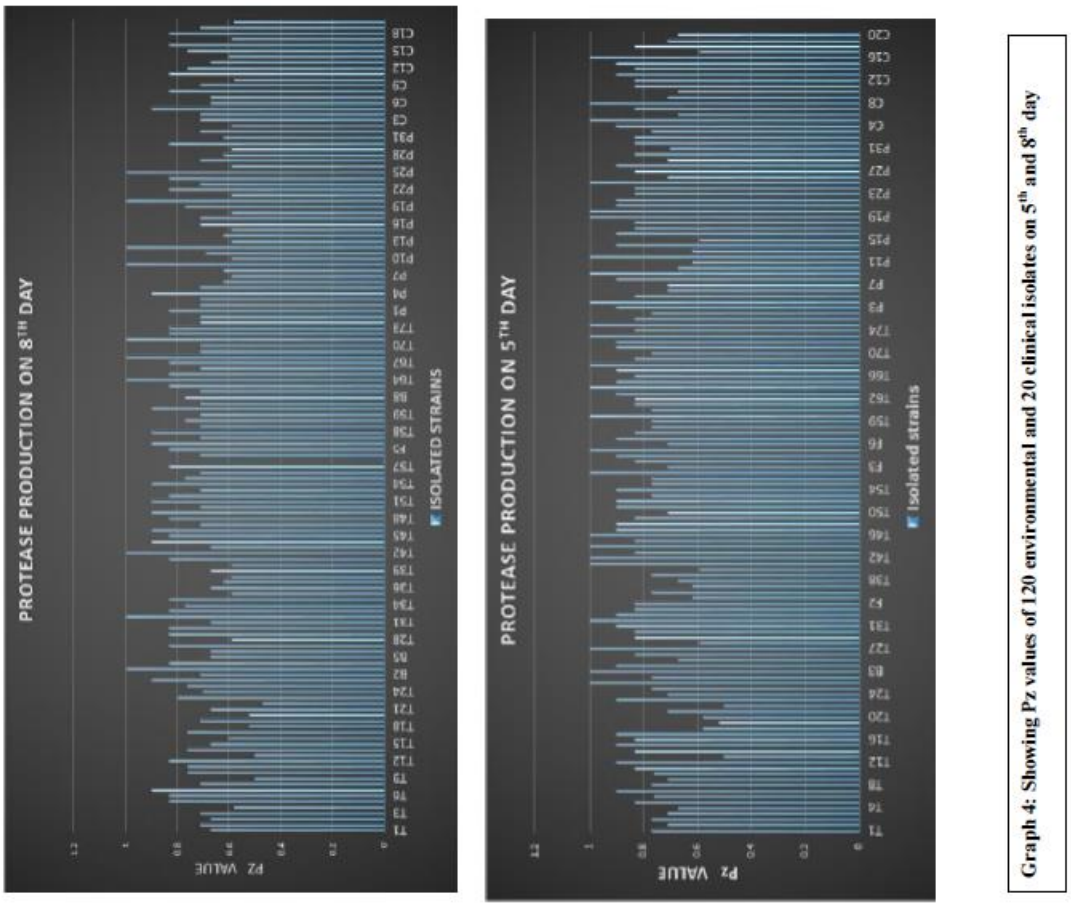


An evaluation of protease activity was done by determining the Pz values of all the environmental and clinical strains. High Pz values (Pz =1) were observed by B1, B3, C-1, T27, T32, T 42, T45, T44, T46, T57, T61, T65, T69, T74, P9, P12, P19, P20, and P25. High protease production was exhibited by strains with isolate number: C-10 (*C. neoformans* var. *grubii*), isolated from a bone cancer patient. C-13 (*C. neoformans* var. *grubii*), isolated from a blood cancer patient, C-20 (*C. neoformans* var. *grubii*), isolated from ovarian cancer patient T-13 (*C. gattii*) isolated from living tree hollow of *Terminalia. arjuna*, T-38 (*C. gattii*), isolated from living tree hollow of *Ficus religiosa* and P-24 (*C. neoformans.* var. *neoformans*), isolated from pigeon excreta. Result was shown in Graph 3 and 4.

An evaluation of DNase activity was done by determining the Pz values of all the environmental and clinical strains. Highest Pz values (Pz =1) on 5th day were observed by the C1, C6, C1, P6, P16, P18, P31, F1, T2, T13, T19, T23, B2, B3, T26, T41, T42, T47, B6, T63, T64, T68, T70. However high DNase production was obtained by strain C-8 (*C. neoformans* var. *grubii*), isolated from a lung cancer patient. C-14 (*C. neoformans* var. *grubii*), isolated from breast cancer patient, C-17 (*C. neoformans* var. *grubii*), isolated from breast cancer patient T32- (*C. gattii*) isolated from living tree hollow of *Peltophorum dulce*, T-35 (*C. gattii*), isolated from living tree hollow of *Ficus religiosa*, and P-24 (*C. neoformans* var. *neoformans*), isolated from pigeon excreta.



Graph 3: Showing Pz values of 120 environmental and 20 clinical isolates on 5th and 8th day



Graph 4: Showing Pz values of 120 environmental and 20 clinical isolates on 5th and 8th day

In this study we examined protease activity of *C. neoformans* and *C. gattii* of clinical and environmental origin by following the method [19], in which extracellular protease activity was examined on 8 clinical (AIDS patients) strains of *C. neoformans* by solid and liquid YCB media supplemented with BSA and polypeptone, which is required as a carbon and nitrogen source, required for efficient and rapid growth in the early stages of development and important for the later efficient production of protease activity.

Identification and discrimination between clinical and environmental isolates of *C. neoformans* varieties can be done on the basis of extracellular enzyme activities [22]. However, we did not find any difference in the enzyme activities by clinical and environmental isolates of *C. neoformans.*

Our results support study who reported that both species produce extracellular DNase, but clinical isolates of *C. neoformans* produce significantly more extracellular DNase than environmental isolates [20]. Extracellular DNase activity exhibits an essential role of in the taxonomic and phylogenetic studies of the genus *Cryptococcus* [11].

In the present investigation, DNase activity was evaluated by clinical and environmental strains of *Cryptococcus* species. DNase activity is responsible for the elevation of the innate immune system in group A *Streptococcus* infection[23]. Similarly, in cryptococcal infection, neutrophils provoke an inflammatory response and found in close association with the infected tissue[24-25].

In the present findings, a statistically no significant Difference was observed in the environmental and clinical isolates *C. neoformans* species complex positive for protease activity using independent 't' test ($p<0.001$). Whereas a significant difference was observed in the clinical strains on the 5th day of DNase production.

CONCLUSION:

Analysis of the results obtained suggests that the capacity of *Cryptococcus neoformans* species Complex to produce protease and DNase may contribute to fungal virulence associated with cryptococcosis. This finding opens a new gateway for the research of new therapeutic strategies associated with these target virulence factors.

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