

Original Research Article

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In-Vitro and *In-Vivo* Pathogenicity Study of *Cryptococcus* Species

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ABSTRACT

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Cryptococcus neoformans encapsulated yeast causes localized or disseminated infections, which may be acute, sub-acute or chronic in nature, in immunocompromized and apparently immunocompetent patients. Unfortunately, knowledge concerning the genetic variability in relation to the pathophysiology of the disease remains controversial. Random Amplification of Polymorphic DNA (RAPD) technique is a novel molecular approach for detection of genetic variability of fungal strains. A RAPD technique using arbitrary primers R28, OPH-02 and OPH-20 clearly indicates genetic variability in *C. neoformans*. To find out relationship between genetic variability and pathogenicity of the *C. neoformans*, we carried out the in-vitro and in-vivo pathogenicity test in mice model, infected with four different RAPD types of Cryptococci. Mice showed a high susceptibility to Pattern I and Pattern IV of *C. neoformans*. This variation in susceptibility is associated with some minute difference in their genotypes but not related with the in-vitro virulence factors i.e. urease activity and melanin production.

Introduction

The member of *Cryptococcus neoformans* species complex, *Cryptococcus neoformans* and *Cryptococcus gattii* are the cause of cryptococcosis of human and animal (Buchanan and Murphy, 1998; Kwon-Chung *et al.*, 2002). Among these, *Cryptococcus neoformans* has been increasingly reported from immunocompromised patients. This encapsulated yeast causes localized or disseminated infections, which may be acute, sub-acute or chronic in nature, in both immunocompromised and apparently immunocompetent patients (Kwon-Chung and Bennett, 1992). The spectrum of predisposing

immunosuppression includes connective tissue disorders, malignancies, diabetes mellitus, steroid-dependent pulmonary disorders, immunosuppressive regimens in relation to transplants, and human immunodeficiency virus (HIV)/Acquired immune deficiency syndrome (AIDS).

India has the second largest burden of Cryptococcosis due to an estimated population of 3.1 million to 9.4 million person living with HIV/AIDS (UNAIDS, 2006). The country has a documented high prevalence (1.7-4.7%) of cryptococcosis in the person

with HIV/AIDS (Kumarasamy *et al.*, 2003; Vajpayee *et al.*, 2003)

Yamamoto *et al.*, (1995) have developed a random amplification of polymorphic DNA (RAPD) techniques using arbitrary primers R28, OPH-02 and OPH-20, which clearly indicates genetic variability in *C. neoformans* (Yamamoto *et al.*, 1995). In this study we compare pathogenicity of RAPD types in both in-vitro and in-vivo model to find out relationship between genetic variability and pathogenicity of organism.

Materials and Methods

Source of the samples

RAPD typing of environmental isolates had already conducted by the Outreach Programme on Zoonotic Diseases (OPZD) at Department of Veterinary Public Health of West Bengal University of Animal and Fishery Sciences. 4 distinct RAPD fingerprint patterns (designated as pattern-I through pattern-IV) were identified by 3 primers R-28, OPH-02 and OPH-20 and preserved in -80°C. Representative strains of each RAPD types were used for pathogenicity study (Fig. 1).

In-vitro pathogenicity study

Biochemical tests

Isolates were also inoculated on C.G.B. (Canavanine- Glycine- Bromothymol Blue) agar media at 25- 30°C up to 5 days to differentiate *C. neoformans* and *C. gattii*. In case of *C. neoformans* var. *neoformans*, colour would not change (Kwon-Chung *et al.*, 1982).

Urease activity

This test was performed in Christensen's urea agar slant which was inoculated with the test

fungus and incubated at 28°C for 7 days. The urease activity usually within 7 days changes the colour of the medium to pink (Cox *et al.*, 2000).

Melanin formation in Bird Seed Agar

Isolates were cultured in Bird Seed Agar (Himedia®). The plates were incubated at 28°C for 2 days and watched for brown pigmentation. All the four RAPD Types are inoculated in Bird Seed Agar (BSA) for the study of Melanin formation.

In-vivo pathogenicity study

Animal experiments were conducted with the approval of Institutional Animal Ethics Committee [IAEC/93/VI(M)] and Institutional Bio-safety Committee [10/IBC/VAS/17] of West Bengal University of Animal and Fishery Science. 50 Swiss male Albino mice with average body weight 25 gm divided in five groups for each RAPD types, CN1 (Pattern I), CN2 (Pattern II), CN5 (Pattern III) and CN9 (Pattern IV) and the last group was used for control and each group having 10 animals. After overnight culture on brain heart infusion broth at 28°C, yeast cells of *C. neoformans* surrounded by intermediate capsule were harvested and then suspended in sterilized Phosphate Buffer Saline (PBS). Infective inoculum was prepared by adjusting number of yeast cells 3.25×10^5 /ml measured in a hemocytometer. Each mouse was intravenously inoculated 0.2ml of inoculum into lateral tail vein. 2 mice from each group were sacrificed after 7th, 14th, 21st and 28th Day of post infection. Mice were euthanized with ketamine @ 200mg/kg b.w. intra-peritoneally and P.M. changes were recorded. The tissues thus collected were kept in vials containing 10 % neutral buffered formalin for histopathological examination. After processing tissue samples were sectioned at 5 micrometer and stained by Hematoxylin and

Eosin (Suvarna *et al.*, 2012). Quantitative assessment of the fungal infection area was performed by observing 10 selected fields randomly at x400 magnification, with the following criteria: 0, no fungi; +, area of fungal infection < 25%; ++, infected area involving 25–50% of the field; and +++, infected area \geq 50% of the field (Surawut *et al.*, 2017).

Results and Discussion

***In-vitro* pathogenicity study**

Biochemical test

All the isolates gave negative test on Canavanine- Glycine-Bromothymol Blue (CGB) Agar (Fig. 2), indicating that isolates were *C. neoformans* var. *neoformans*.

Urease

All RAPD Types were urease positive in Christensen's medium and changed colour of the medium from yellow to bright pink (Fig. 3).

Melanin production

Melanin production was assessed by verifying the extent of brown pigment formation was confirmed in all the strains. Melanin production did not differ significantly among the all RAPD types (Fig. 4; Table 1).

***In-vivo* pathogenicity study**

Clinical signs

Mice showed clinical signs of disease like hunching, panting, weight loss, reduced food/water intake and inactivity between day 7 and day 9 post infections.

Gross morphology and necropsy finding

No abnormality was detected when mice were sacrificed after one week of inoculation. But Carcass was severely dehydrated, emaciated. Doming/Enlargement of head were observed 14 days after inoculation (Fig. 5). Sever congestion of meninges and nodular opaque regions were detected in kidney after two and three weeks of inoculation (Fig. 6).

Histopathological studies

Histopathologically, the brain and liver were severely affected with the fungus. In the brain cysts with Cryptococci continued to increase until the end of the experiment (Fig. 7 and 8). On the other hand, numerous mononuclear cells infiltrate into the live and produced several foci of granulomatous lesion with Cryptococci after 14 days of inoculation (Fig. 9 and 10). Quantitative measurement of the pathogenicity were performed by observing cyst and granuloma formation in liver and brain on 7th, 14th, 21st and 28th days. The majority of these strains were environmental isolates. This study revealed that CN1 and CN9 both of the isolates produced marked degree of cyst and granuloma formation in brain, liver (Table 2).

The frequency and mortality due to opportunistic mycoses are increasing among patients with AIDS, hematologic malignancies, and transplants. This emphasized the importance of understanding the pathophysiology of this emerging fungal pathogen. Unfortunately, knowledge concerning the genetic variability in relation to the pathophysiology of the disease remains controversial. RAPD technique is a novel molecular approach for detection of genetic variability of fungal strains.

Table.1 RAPD fingerprint pattern of the 14 isolated *C. neoformans* strains. (Kindly supplied by Outreach Programme on Zoonotic Diseases, Department of Veterinary Public Health, WBUAFS)

Strain no.	RAPD profile			
	Primer OPH-20(A)	Primer OPH-02(B)	Primer R-28(C)	Pattern
CN1	a	i	1	I
CN2	b	ii	2	II
CN3	a	i	1	I
CN4	c	iii	3	III
CN5	c	iii	3	III
CN6	b	ii	2	II
CN7	-	-	3	IV
CN8	a	i	1	I
CN9	-	-	3	IV
CN10	b	ii	2	II
CN11	-	-	3	IV
CN12	a	i	1	I
CN13	b	ii	2	II
CN14	a	i	1	I

Table.2 Quantitative assessment of Cryptococcal infection in brain and liver X400

Days		7 th day	14 th day	21 st Day	28 th Day
Group No.					
CN1	Brain	0	+	++	+++
	Liver	0	+	+++	+++
CN2	Brain	0	+	+	+
	Liver	0	+	+	+
CN5	Brain	0	+	+	+
	Liver	0	+	+	+
CN9	Brain	0	+	++	+++
	Liver	0	+	++	+++

Fig.1 Primer OPH-20 revealed major differences in banding patterns ranging between 1500bp and 400bp, which distinguished the profiles a, b and c. Primer OPH-02 revealed 3 different profiles in banding patterns ranging between about ranging between about 2000bp and 400bp, which distinguished the profiles in three categories namely i, ii and iii. Primer R-28 revealed 3 different profiles in banding pattern ranging between 1500bp and 200bp, which distinguished the profiles in 1,2 and 3 categories. (Kindly supplied by Outreach Programme on Zoonotic Diseases, Department of Veterinary Public Health, WBUAFS)

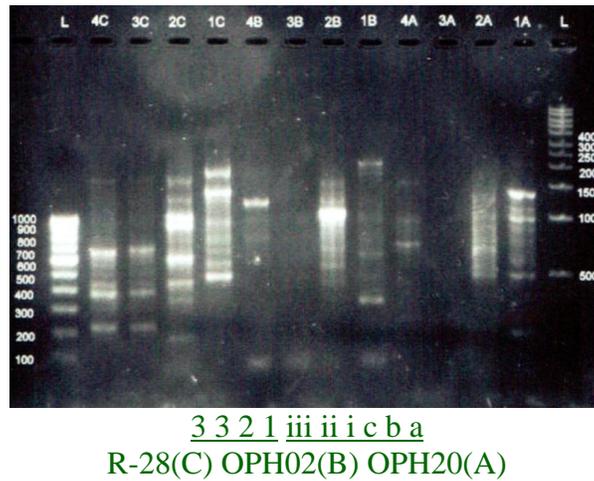


Fig.2 Growth on CGB agar. Showing colonies of *C. neoformans* with no colourchange of media(left side) and uninoculated control (right side)



Fig.3 Urease Activity of *C. neoformans*. First tube from left side used as control and other tubes showing positive Urease activity by developing pink colour

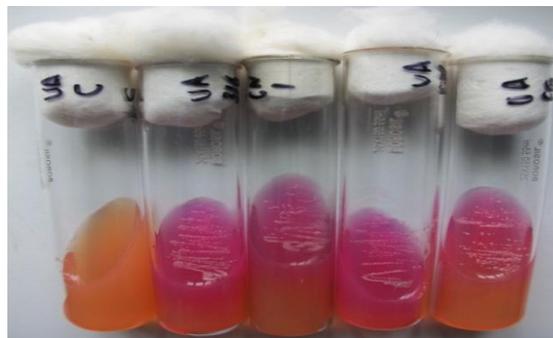


Fig.4 Brown coloured colonies of *C. neoformans* CN9 in Bird Seed Agar



Fig.5 Mice showing marked degree of chacexia and doming of head 14 days after Inoculation of *C. neoformans*



Fig.6 Marked degree of congestion of meninges 14 days after inoculation of *C. neoformans*



Fig.7 Cyst formation in brain 14 days after inoculation. H and E. X100

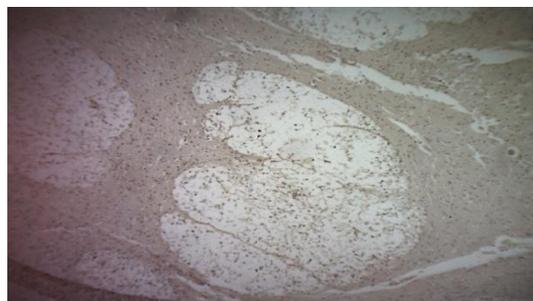


Fig.8 Cyst containing numerous yeast cells(arrow) in the brain 14 days after inoculation of 3.25×10^5 cryptococci. Sothgate's mucicarmine stain, X400

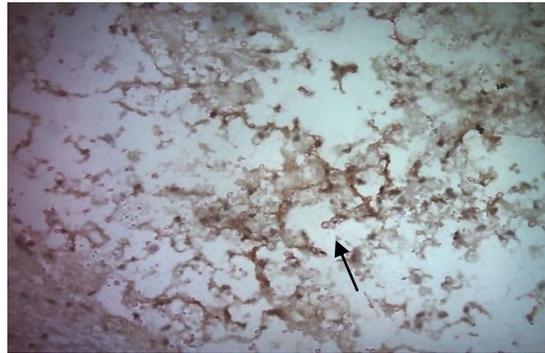


Fig.9 Granuloma(arrow) formation in liver, H andE, X100

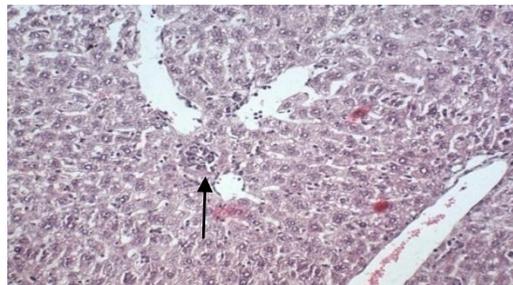
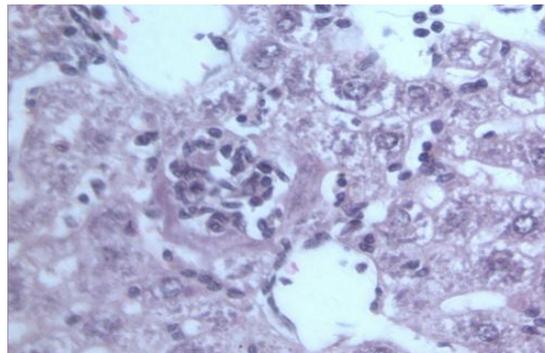


Fig.10 Granuloma in Liver 14 days after inoculation, HandE, X400



To elucidate this of the *C. neoformans*, we investigated the biological characteristics of pathogens and the pathophysiology of mice infected with four different RAPD types of *Cryptococci*. Urease catalyzes the hydrolysis of urea to ammonia and carbamate. Under physiological conditions, this reaction can result in an increase in pH. Urease activity has been found in several bacteria, fungi, and plants and has been shown to be an important

pathogenic factor (Eaton *et al.*, 1991). Melanin was found to be highly effective in protecting fungal structures from lysis or decomposition by natural communities of microorganisms (Kuo and Alexander, 1967).

In vitro results of urease activity, melanin production showed that all the tested RAPD types expressed these virulence factors, although to different extents. In the present

histopathological study CN1 and CN9 showed a higher degree of pathogenicity and also provided evidence again for the important role of cell-mediated immunity to experimental cryptococcosis. In the liver, yeast cells inoculated intravenously into mice were engulfed by macrophages of the organ as soon as carried to it by blood stream. Miyaji and Nishimura suggested that these macrophages might not have a fungicidal ability against them. In order to destroy them the cell-mediated immunity had to be exerted (Miyaji and Nishimura, 1981). In the liver granuloma was the specific lesion of cell-mediated immunity induced by *C. neoformans* and yeast cells were destroyed in the granulomatous tissue. This study also suggested that major histopathological features of the central nervous system were cystic and cysts with cryptococci expanded and continued to increase until the end of the experiment. In this study the number of granulomas and cryptococci reached a peak on the 14th day in the mice and thereafter decreased, except CN1 (Pattern I) and CN9 (Pattern IV), which produced significantly higher level of infection in liver and brain even after 28 days.

Overall, these findings suggest that RAPD types belong to isolates *C. neoformans* have different pathogenicity in murine model. Mice showed a high susceptibility to Pattern I and Pattern IV of *C. neoformans*. This variability in susceptibility is associated with some minute difference in their genotypes but not related with the in-vitro virulence factors i.e. urease activity and melanin production.

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