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Original Research Article

Serotype, Mating Type and Molecular Epidemiology of Environmental Cryptococcus Neoformans Isolated from Selected Areas of Tamil Nadu, India

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ABSTRACT

Objectives: To isolate *Cryptococcus neoformans* from pigeon droppings collected in different regions of South India and their serotyping and molecular characterization using Random Amplified Polymorphic DNA (RAPD) fingerprinting analysis.
Methods: This prospective study comprised of 118 samples collected during 12th September 2012 to 21st March 2015 from different regions in Tamil Nadu, India and were examined for the presence of *C. neoformans* using Bird Seed Agar (BSA) medium. The serotypes of the isolated fungal strains were determined by employing multiplex PCR, using primers specific for laccase gene (*LAC1*) and capsular gene (*CAP64*). The molecular and mating types of the isolated *C. neoformans* were determined by PCR fingerprint analysis with the help of mini and micro specific satellite sequences respectively.
Results: From the 118 pigeon dropping samples collected, 32 distinctive mucoid yeast colonies were retrieved and morphologically characterised as *C. neoformans*. This study confirms the serotypes *C. neoformans* var. *grubii* (serotype A) and var. *neoformans* (serotype AD/D) were prevalent in Tamil Nadu, India. PCR-fingerprinting pattern based using M13 and $(GACA)_4$ revealed the distribution of VNI, VNII, VNIII and VNIV molecular types in the isolates. Based on their relationships in genotypes, a dendrogram was also derived using Neighbour joining algorithm.
Conclusion: The findings of the present investigation elucidated the molecular diversity of the *C. neoformans* strains in Tamil Nadu and also provide evidence for genetic relatedness of the isolates in the environment.

Key Words: Serotypes, genotypes molecular types, *C. neoformans*, PCR.

INTRODUCTION

Cryptococcus neoformans is an encapsulated basidiomycete, which causes opportunistic fungal infections in both immunocompromised as well as immunocompetent patients, leading to endangering medical conditions such as cryptococcosis and cryptococcal meningitis. Cryptococcosis is a significant incidence of cryptococcal infection or inflammation among immune expressed hosts and seemed to be responsible for

high mortality and morbidity rates among AIDS/HIV infected patients. On the other hand, cryptococcal meningitis is one of the dangerous, life-threatening cerebral infections affecting the central nervous system (CNS). Human cryptococcal infections are developed by inhaling infectious propagules including, airborne desiccated yeast cells or basidiospore from environmental sources. [1-3]

The prevalence of *C. neoformans* and its related infection differs according to the epidemiology, molecular profiles, population, morphological and physiological characterisation, geographic distribution and the period of investigation studied. *Cryptococcus* spp. was classified into three varietal types and five serotypes; the first being *C. neoformans* var. *grubii* (serotype A), the second *C. neoformans* var. *neoformans* (serotype AD/D) and the third type being *C. neoformans* var. *gattii* conventionally regarded as *C. gattii*, which is marked by serotypes B and C. The first and second varieties were being characterised as haploid isolates, whereas the third was recorded as diploid. [4-5] *Cryptococcus gattii* was mostly identified in the tropical and subtropical regions leading to pulmonary cryptococcomas. However, more than 90% of global fungal infections were contributed by the haploid strains of *C. neoformans* var. *grubii* possessing the serotype A capsular epitope. [5] In *C. neoformans*, the virulence (extremely pathogenic) phenotype may feature recessive trait expressed only in the haploid strains while in the diploid strains, the phenotype may or may not be shown based on the alleles found in the specific strain. [6]

In the environmental surroundings, isolates of *C. neoformans* are frequently isolated in soil surfaces, decayed wood substrates, hollow trees, plants and bird's excreta. Due to their high body temperature, pigeons and many such birds do not get infected but can harbour the fungal cells. [7] However, the excrement of these reservoir birds is a natural fortification and ideal medium for the environmental survival of *C. neoformans* and they serve in the dissemination of the yeasts cells. [8]

Detailed investigations on epidemiological studies of *Cryptococcus* are vital to know the biology and ecology of this species so as to enhance the management strategies against the infections caused by *Cryptococcus* species. Many molecular typing methods are being employed for the sporadic and epidemiological investigation of environmental and/or clinical isolates of *C. neoformans*, such as, Karyotyping, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), PCR Fingerprinting, Amplified Fragment Length Polymorphism (AFLP) and Mulitlocus sequence typing (MLST). [9-18]

In India, although *C. neoformans* is implicated as a primary source for causing mycotic infection in AIDS patients, the details on the molecular epidemiology of all isolates is not very clear. The Indian subcontinent featuring the tropical climate serves as a suitable platform for the environmental prevalence and existence of *C. neoformans*. The prevalence of extensive forms of disease presentations and symptoms in India reveal the possibility of several strain variations among the species, *C. neoformans*. In addition, the distribution of fungal serotypes in the environmental isolates found in India were different from those available in other areas with serotype A being the majority and serotype B being the minority significantly among the Indian isolates. [19] Since India is one of the densely populated countries in the world experiencing a considerable increase in the prevalence of AIDS and cryptococcosis, the necessity for a comprehensive analysis of the *C. neoformans* is much recommended. Thus, the present study was attempted to examine the molecular epidemiology of *C. neoformans* isolated from the pigeon

droppings. In light of this, we also analysed the serotype, molecular and mating type of the environmental isolates of *C. neoformans* isolated from different regions of Tamil Nadu using PCR-fingerprinting analysis.

MATERIALS AND METHODS

Study Area: In this prospective study, four main districts were chosen: Chennai, Vellore, Salem and Tiruchirapalli or Trichy. Chennai (13.04°N; 80.17°E) lies on the thermal equator zone at 6-60 m above the sea-level with a mean annual temperature of 32.8°C and relative humidity of 60%, and located on the southeast coast of India and northeast corner of Tamil Nadu. Vellore, located in the north eastern part of Tamil Nadu (12.92°N; 79.13°E), lies 220 m above the sea-level with a mean annual temperature of 33.4°C and relative humidity of 40-63%. Salem, located in the southwest district of the capital city Chennai (11.66°N; 78.14°E), is 278 m above the sea-level and records a mean annual temperature of 33.8°C and relative humidity of 72%. Tiruchirapalli is located in the central south eastern part of India, almost at the geographic centre of Tamil Nadu (10.80°N; 78.68°E.), where the altitudes vary between 80 and 88 m above the sea-level with a mean annual temperature of 28.9°C (Figure 1).

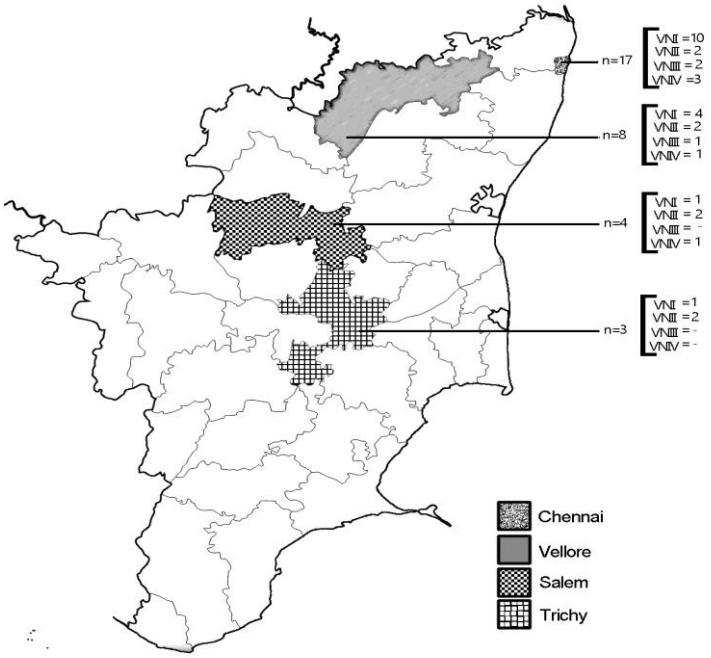

Figure 1: The district wise distribution of cryptococcal isolates involved in this study

Cryptococcal Strains: For the isolation of *C. neoformans* strains, a total of 118 pigeon dropping samples were collected from various environmental sources during 12th September 2012 to 21st March 2015 from the four different regions of Tamil Nadu, India. The sources include residential buildings, bird shops, food markets, schools, colleges, playgrounds, religious places, municipal buildings, hospitals, parks, plazas and cinema theatres. Information about the sample collection sites are summarised in Table 1. The samples were collected in sterile zip-lock polyethylene bags, labelled, brought to the laboratory and processed immediately.

Standard Strains: The strains WM148 (VNI-A), WM626 (VNII-A), WM628 (VNIII-AD), WM629 (VNIV-D) were used as standard reference strains for RAPD and PCR-fingerprinting analysis of *C. neoformans*. [14]

Fungal Isolation: *Cryptococcus neoformans* were retrieved from the droppings of pigeon according to the protocol described by Casali *et al.* [12] a defined volume of pigeon excreta (1 g) collected from different locations was weighed aseptically and transferred to Erlenmeyer flasks containing 10 mL of Phosphate buffer saline (PBS) (Sigma-Aldrich, India) amended with chloramphenicol (200 mg/L). The mixture was thoroughly homogenised by vortexing and later allowed to stand for 30 mins at 37^{o}C. After filtration trough gauze, 0.1 mL aliquots of 10^{-1} dilution was inoculated onto Bird Seed Agar (BSA) medium and incubated at 32°C for 8 days. The contamination of each sample was confirmed by the existence of *C. neoformans* in BSA medium after 5 to 8 days of incubation. The highest and lowest level of contamination by the species *C. neoformans* in the examined zones was depicted with respect to the percentage of positive samples found and then the strains were sub-cultured on Sabouraud's dextrose agar (SDA) slants and stored at -4°C.

Morphological Characterisation: Morphological identification of *Cryptococcus neoformans* was done based on the formation of yeast-like colonies showing a cream to dark brown homogeneous pigmentation with smooth and mucoid-like appearance. The suspected colonies were selected and the pure cultures were maintained in SDA medium. Well-grown cryptococcal cells were examined microscopically with Indian ink to investigate the presence of capsule. The distance from the cell wall until the capsule's outer margin as well as the cell diameter (leaving the capsule) were precisely measured with an eyepiece grid having a resolution of 0.5 m. All the strains were then subjected to urease production test and the positive isolates were identified by their carbohydrate fermentation and temperature tolerant ability. [20]

Variety Typing Using CGB Medium: Variety typing of the isolated strains were done by L-canavanine glycine-bromothymol blue (CGB) medium, developed by Kwon-Chung *et al* [21] which differentiates the species *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotypes A and AD) from *C. gattii* (serotypes B and C). Isolates pre-grown in SDA medium for 48 h were streaked onto CGB medium and incubated at 32°C for 24 h. A colour change from light yellow to deep cobalt blue was remarked as the positive outcome with respect to CGB test, confirming the presence of *C. gattii* (Serotype B/C). If there was no change in colour from light yellow or green, it indicated the presence of *C. neoformans* var. *neoformans* and var. *grubii* (Serotype A/D).

Preparation of DNA: Genomic DNA was isolated from the fungal strains using the protocol described by Meyer *et al.* [14] Temporarily, the isolated and standard reference strains were grown on SDA medium for 3 days. A loopful of *C. neoformans* culture was scraped from SDA medium and suspended in 250 µL TE buffer (0.5 mM EDTA; 50 mM Tris-HCl; pH 8.5) in an Eppendorf tube and later kept in a water bath for 20 min at 85°C. After vortexing, the suspension was centrifuged for 15 min at 15,000 rpm and the supernatant was mixed well with 0.5 mL of extraction buffer (0.25 mM EDTA, 1mM Tris-HCl, 1.25 M NaCl, pH 8.5) and 0.1 mL of 10% sodium dodecyl sulphate. The yeast cells were then disrupted by vortex and genomic DNA was extracted thrice using phenol: chloroform: isoamyl alcohol (24:24:1). The developed aqueous phase was transferred to a fresh Eppendorf tube and the DNA was precipitated using absolute ethanol. The precipitated DNA was resuspended in 0.5 mL TE buffer, stored at -20°C and quantified using agarose gel electrophoresis.

Determination of serotype by multiplex PCR: PCR amplification of *LAC1* and *CAP64* genes was performed using the

method described by Ito-Kuwa *et al.* [22] four primers specific for *LAC1* gene and a pair of primer specific for *CAP64* gene was designed at the concentration of 10 µM (Table 2). [21] For multiplex PCR, 2 µL of each primer was added to a total reaction mixture of 50 µL containing genomic DNA. A ratio of 1:0.25 *LAC1* and *CAP64* gene was chosen because amplification with capsule gene primers proceeded more efficiently than laccase specific primers for multiplex PCR. Three sets of amplification reactions were performed with 4 primers of *LAC1* in the first set, 2 pairs of *CAP64* primers in the second set and the third set with all 6 primers. The band patterns of the amplified products were electrophoresed and the gels were then stained with ethidium bromide and visualised under UV trans-illuminator.

PCR Fingerprinting Analysis: In general, for the PCR fingerprint analysis, minisatellite-specific core sequence of the wild-type phage M13 (5'-GAGGGTGGCGGTTCT-3') and oligonucleotides of the microsatellite-specific sequence $(GACA)_4$ were used as single primer in the PCR procedure, as described in the modified methods of Meyer et al. [14] The 50 µL reaction mixture contains, 40.5 µL of sterile deionized water was added to a tube containing 25 ng of DNA, 1x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin) (Sigma-Aldrich, India), 0.2 mM of each dNTP (Sigma-Aldrich, India), 20 ng primer and 2.5 µL Taq DNA Polymerase (15 U μL^{-1} (Sigma-Aldrich, India). PCR reaction was performed in thermal cycler (Bio-Rad, USA) using the following reaction conditions: initial denaturation at 94°C for 3 min followed by 40 cycles of denaturation (94°C, 30 s), annealing (50°C, 1 min), extension (72°C, 1.5 min) and a final extension step at 72°C for 6 min. The PCR amplicon were separated by electrophoresis in 1.5% agarose gel at 100V for 30 min and the DNA bands were subjected to visualisation under UV trans-illuminator. A 100kbp molecular-sized marker was kept in every gel as a control.

Determination of Mating Type: Mating type of all the positive strains were identified through PCR analysis using two primers specific for *MATα* and *MATa*, as shown in Table. 3 according to the procedure described by Chaturvedi et al. [23] PCR amplification reactions were performed in a final volume mixture of 25 µL using (Bio-Rad, USA) as described previously. Amplified PCR products were electrophoresed on 1.5 % agarose gels in 1X TBE buffer at 100 V for 30 min and visualised under UV trans-illuminator.

Statistical Analysis: Statistical analysis was used to determine the genetic relatedness of closely related isolates. They were performed by neighbour-joining algorithm method using the SPSS software package version 20.0 (IBM, Armonk, New York).

RESULTS

Cryptococcus neoformans is an opportunistic, life-threatening yeast pathogen causing fungal-type infections among the immunocompromised as well as competent hosts. Birds such as pigeon excrements are found to be a major carrier of *C. neoformans* and leading to its related diseases. India is a country which is now in the midst of an epidemic of AIDS-related cryptococcosis. In the present investigation, serotypes, fungal pheromones and the genetic diversity of *C. neoformans* isolated from pigeon faeces were analysed using RAPD and PCR-fingerprinting. For the purpose, 118 samples of pigeon droppings were collected from four different regions of Tamil Nadu. The fungal contamination in the pigeon droppings were enumerated using BSA medium. Out of the 118 collected samples, 32 positive strains of *C. neoformans* were retrieved. The species was characterised by the formation of dark-brown coloured yeast-like colonies on BSA medium. The isolation of all *C.*

neoformans strains was done from the pigeon droppings especially laid on residential buildings and public markets, and the prevalence rate was around 27.1% (32 out of 118 samples) among the isolates. There was a significant variation in the segregation percentage among the isolates collected from the four different regions of Tamil Nadu (Table 1).

Table 1: Origin, serotype, molecular type, and mating type of *C. neoformans* strains used in this study

S. No.	Strain	Isolation Source	Origin of specimens	Variety	Molecular Type*	Serotype	Mating type
1.	WM148	Reference	Clinical	*C. neoformans. grubii*	VNI	A	α
2.	WM626	Reference	Clinical	*C. neoformans. grubii*	VNII	A	α
3.	WM628	Reference	Clinical	*C. neoformans. neoformans*	VNIII	AD	α
4.	WM629	Reference	Clinical	*C. neoformans. neoformans*	VNIV	D	α
5.	RNE03	Chennai Zone	Residential buildings	*C. neoformans. grubii*	VNI	A	α
6.	RNE07	Chennai Zone	Residential buildings	*C. neoformans. grubii*	VNI	A	α
7.	SNW13	Chennai Zone I	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNI	A	α
8.	SNW19	Chennai Zone I	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNI	A	α
9.	SNE20	Chennai Zone I	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNI	A	α
10.	SNE23	Chennai Zone I	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNI	A	α
11.	SNW25	Chennai Zone I	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNI	A	α
12.	SNW31	Chennai Zone II	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNII	A	α
13.	RPPNE32	Chennai Zone	Religious places	*C. neoformans. neoformans*	VNIV	D	α
14.	RCNE41	Chennai Zone	Residential buildings	*C. neoformans. neoformans*	VNIII	AD	α
15.	RCNE46	Chennai Zone	Residential buildings	*C. neoformans. neoformans*	VNIV	D	α
16.	SCNE53	Chennai Zone II	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNII	A	α
17.	SCNE55	Chennai Zone III	Schools, colleges and playgrounds	*C. neoformans. neoformans*	VNIV	D	α
18.	PCW56	Chennai Zone III	Parks, plazas and cinema theatres	*C. neoformans. neoformans*	VNIII	AD	α
19.	PCW57	Chennai Zone III	Parks, plazas and cinema theatres	*C. neoformans. grubii*	VNI	A	α
20.	PCNE58	Chennai Zone III	Parks, plazas and cinema theatres	*C. neoformans. grubii*	VNI	A	α
21.	PCNE60	Chennai Zone III	Parks, plazas and cinema theatres	*C. neoformans. grubii*	VNI	A	α
22.	BWNE67	Vellore Zone I	Bird shop and food market	*C. neoformans. neoformans*	VNIV	D	α
23.	BWNE73	Vellore Zone I	Bird shop and food market	*C. neoformans. neoformans*	VNIII	AD	α
24.	RNE74	Vellore Zone I	Residential buildings	*C. neoformans. grubii*	VNI	A	α
25.	RNE75	Vellore Zone I	Residential buildings	*C. neoformans. grubii*	VNI	A	α
26.	SNW83	Vellore Zone III	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNI	A	α
27.	SNE87	Vellore Zone III	Schools, colleges and playgrounds	*C. neoformans. grubii*	VNI	A	α
28.	RPPNE88	Vellore Zone	Religious places	*C. neoformans. grubii*	VNII	A	α
29.	RPPNE90	Vellore Zone	Religious places	*C. neoformans. grubii*	VNII	A	α
30.	RPPNE95	Salem Zone II	Religious places	*C. neoformans. grubii*	VNII	A	α
31.	RPPNE96	Salem Zone II	Religious places	*C. neoformans. grubii*	VNII	A	α
32.	PCW99	Salem Zone II	Parks, plazas and cinema theatres	*C. neoformans. grubii*	VNI	A	α
33.	PCW107	Salem Zone III	Parks, plazas and cinema theatres	*C. neoformans. neoformans*	VNIV	D	α
34.	BWN1110	Trichy Zone I	Bird shop and food	*C. neoformans. grubii*	VNII	A	α
35.	BWN111	Trichy Zone I	Bird shop and food	*C. neoformans. grubii*	VNII	A	α
36.	RPWSW115	Trichy Zone I	Religious places	*C. neoformans. grubii*	VNI	A	α

*Molecular type as determined by PCR-fingerprinting with the minisatellite M13 and microsatellite specific primer $(GACA)_4$

The isolated strains were subjected to staining with Indian ink for further morphological characterisation and for the existence of capsule. *Cryptococcus neoformans* had large polysaccharide capsule which was confirmed by the development of halo zone that was clearly seen around the cell on very dark

background. All of the 32 suspected isolates possessing distinct, wide gelatinous capsule were confirmed as *C. neoformans*. The capsule size of all positive isolates ranged from 1.2 to 3.8 µm when measured under the microscope through Indian ink suspension. The capsule size of the derived isolates was compared with the capsule size of reference strains (2.2 µm ± 0.4 versus 2.8 µm ± 0.4; P = 0.2). It was found that no considerable decrease in capsule size was documented for sequentially isolated positive strains collected from different areas. Further studies on biochemical characteristics, urease production and carbohydrate fermentation revealed that all the isolates were urease positive and possessed carbohydrate fermenting ability along with high temperature tolerance.

In the present investigation, L-canavanine Glycine - bromothymol Blue (CGB) agar medium was used for the variety typing of isolates. The results revealed that, all the 32 isolates belonged to *C. neoformans* var. *grubii* and var. *neoformans,* and there is no observance of *C. gattii* in the positive isolates, since it is only endemic to tree samples. The 32 isolated *C. neoformans* strains exhibited less growth with mild change of colour to yellow and showed smooth, mucoid colony morphology.

Table 2: Primers used for the amplification of *LAC1* and *CAP64* genes

LAC1	Primer Sequences
	5′-GGAACAGCAACCACACTACTG-3′
	5′-CATATTGGGTGGCATCTTACTGAGGGA-3′
	5′-CCAGGGAACATGTTGTTGAC-3′
	5′-GTTGTGGAAGGCAAAGAAAC-3′
CAP64	5′-GCCAAGGGAGTCTTATATGG- 3′
	5′-GCAAAGGGTTCACCAAATCG- 3′

Table 3: Primers used for the amplification of *MAT α* and *MAT genes*

	Primer Sequence
MAT *α*	5′-CTTCACTGCCATCTTCACCA-3′
	5′-GACACAAAGGGTCATGCCA-3′
MAT a	5′-CGCCTTCACTGCTACCTTCT-3′
	5′-AACGCAAGAGTAAGTCGGGC-3′

Serotyping: The genetic structure of *C. neoformans* is very complex and hence, novel and specific primers must be designed for RAPD mediated serotyping. Therefore, in the present investigation, *LAC1* and *CAP64* genes were used for serotyping of *C. neoformans* strains. After amplification, three DNA bands measuring 0.96, 0.70 and 0.25 kb in size were obtained for serotype A strains. On the other hand, two DNA bands were produced by serotype D and AD strains measuring 0.86 and 1.1 bp in size respectively.

PCR Fingerprint Analysis: In order to examine the genetic relatedness of the isolated cryptococcal strains, M13 minisatellite and $(GACA)_4$ microsatellite specific primers were employed to amplify the specific DNA sequence within the cryptococcal genome. In spite of the greater discriminatory power, the PCR pattern analysis failed to detect the variability, rather they were able to discriminate from the reference strains and produce molecular profiles according to phage M13 core sequence primer amplification for the isolated strains. The PCR fingerprinting analysis generated the genotypes of the 32 positive strains and they were grouped into 4 main molecular types (genotypes with >92% of similarity). About 24 out of the 32 environmental isolates were proved to belong to the group VNI and VNII (*C.* var. *grubii*, serotype A) and 8 isolates belonged to group VNIII and VNIV (*C.* var. *neoformans*, serotype D along with 3 hybrid serotype AD). Although VNI was the most usual molecular type found among the Indian environmental isolates (50%), less frequent VNII, VNIII, VNIV were also found (Table 1). In the present study, PCR fingerprint was used to differentiate the molecular profiles of the isolates by comparing the bands with the standard reference strains.

Cluster analyses of the isolated strains combined the environmental strains possessing same molecular type. The overall similarity of the Indian isolates was >92%, based on the combined fingerprint data set (Figure 4). Similarly, dendogram analysis obtained with (5'-

GAGGGTGGCGGTTCT-3') and $(GACA)_4$ revealed an overall high heterogeneity among the fungal isolates (32 strains) from Tamil Nadu. The mean coefficient value of the strains was 0.92 ±0.08. About four major groups were formed as a result. The group I comprised of isolates RNE03, RNE07, SNE20, SNE23, SNW25, PCW57, PCNE58 and PCNE60 with major bands of approximately 1250 and 960 bp size (Figure 2). The two major bands of group II (1330 and 530 bp) consisted of isolates BWNE73, RNE74, RNE75, SNW83, RCNE41, PCW56 and SNE87 (Figure 3). Finally, the group III and IV consists of 4 isolates that showed 100% similarity with PCR fingerprinting. All strains in group I were isolated from the pigeon droppings collected from Chennai and Vellore, while strains in group II were isolated from Salem and Vellore pigeon droppings and group III and IV strains were collected from the pigeon droppings found in Salem and Trichy.

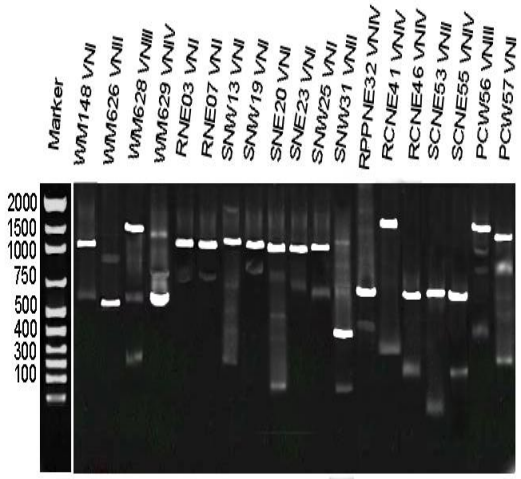

Figure 2: PCR-fingerprinting profiles of environmental *C. neoformans* isolates generated with M13 and (GACA) $_4$ primers.

Determination of Mating Type by PCR: As expected, all of the Indian serotype A and D isolates exhibited MAT α mating type. The MAT α and MAT a specific primers pair was used for amplification of specific fragments of 180 and 145 bp, respectively. Epidemiological analysis for determination of mating type proved 100% existence of MAT α in all the strain isolated zones.

Lane 1: Molecular weight marker; Lane 2-5: Molecular types VNI (A), VNII (A), VNIII (AD) and VNIV (D); Lane 6 -12 & 20: *C. grubii* strains from Chennai zone I & II(VNI-A); Lane 13 & 17: *C. grubii* strains from Chennai zone II (VNII-A); Lane 14–16 & 18: *C. neoformans* strains from Chennai zone II and zone II (VNIV-D); Lane 19: *C. neoformans* samples collected from Chennai zone III (VNIII-AD).

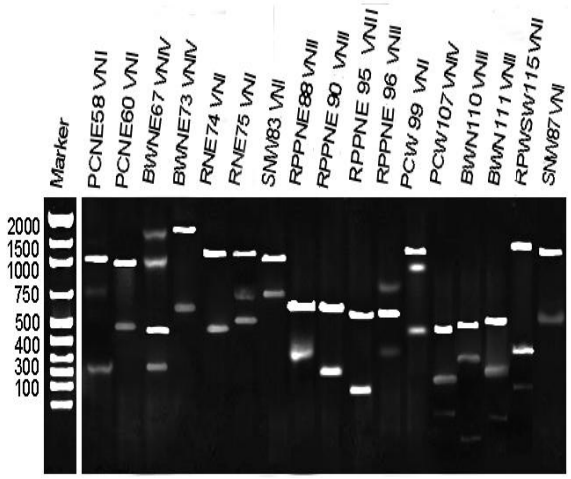

Figure 3: PCR-fingerprinting profiles of environmental *C. neoformans* isolates generated with M13 and $(GACA)_4$ primers

Lane 21: Molecular weight marker; Lane 22 & 23: *C. grubii* strains from Chennai zone III (VNI-A); Lane 26–28: *C. grubii* strains from Vellore zone I & III (VNI-A); Lane 33, 37 & 38: *C. grubii* strains from Salem zone II, Trichy zone I and Vellore Zone-III– (VNI-A); Lane 24, 25, 34: *C. neoformans* strains from Vellore zone I and Salem zone III (VNIV-D); Lane 29-32, 35 &36: *C. grubii* strains from Vellore Zone III, Salem zone II and Trichy zone I (VNII-A).

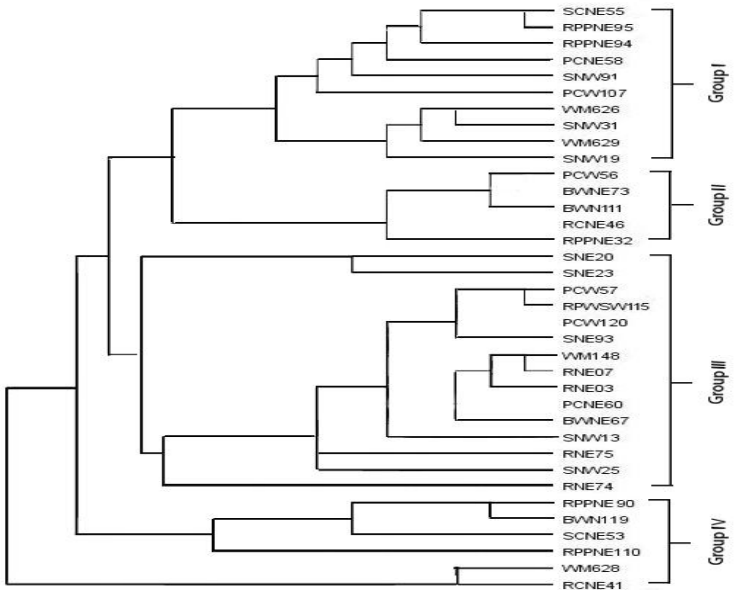

Figure 4: Dendrogram based on relationship between the obtained genotypes from the reference strains studied using Neighbour joining algorithm. Each major group was determined when percentage was >92% of similarity.

DISCUSSION

Cryptococcus neoformans is an opportunistic fungus slowly emerging as a 'champion fungus'. However, in India, particularly in Tamil Nadu, only less has been understood regarding the epidemiology of cryptococcus related diseases. The available information about molecular epidemiology is scanty and hence more studies are required to expose the nature and distribution of eight major molecular types common among the Indian environmental isolates. Very few studies have been reported on the examination of *C. neoformans* genotype, serotype, mating type and its distribution in India. [24-26] Hence, molecular typing and serotyping has been considered as one of the constructive methods for knowing the epidemiological characteristics of these fungal isolates, thereby helping in infection control, disease prevention and therapeutic regulations. Thus, the prime goal of this study is to identify the mating type, molecular type and serotype of *C. neoformans* isolates collected from the pigeon droppings of different regions of Tamil Nadu, India. There is no diagnostic value for serotyping, but it aids in choosing the type of medical treatments with respect to their serotypes. On the other hand, molecular typing helps to explore the pathogenicity and virulence of microorganisms.

In the earlier study carried out by Gokulshankar *et al* [27] out of 222 samples of *C. neoformans* collected from various avifauna around Chennai regions, 60 isolates were determined as *C. neoformans* var. *neoformans*. Similarly, in a study lead by Girish Kumar et al [28] about 12 (33.3%) out of 40 tree samples isolated from South India showed positive result for the presence of *C. neoformans* var. *neoformans*. It is likely that the low rate of isolation of *C. neoformans* in the present investigation was due to the availability of low sample quantity and elevated environmental temperatures in the sampling regions. In this study, *C. neoformans* var. *gattii* was not isolated indicating the fact that this variety may not be prevalent in the specified geographical region (Tamil Nadu, India) and that this variety is not usually an inhabitant of pigeon droppings. The current findings are supported by the earlier report of Gokulshankar *et al.* [27] However; further more extensive studies are needed to confirm the prevalence of variety *gattii* in this region.

PCR-RAPD technique was employed with the aim of differentiating serotypes and molecular types of *C.*

neoformans isolated for this study. RAPD analysis serves as a reliable and sensitive method for molecular typing of *C. neoformans*. The appropriateness of RAPD analysis was proved by revealing its capacity to differentiate among closely associated isolates within the stipulated geographic areas. [29-31] The genetic structure of the fungal species, *C. neoformans* is really complicated and hence, the application of new primers, including *LAC1* and *CAP64*, and latest molecular tools used in this study are crucial to identify their serotype. All the cryptococcal isolates investigated in this research were recovered from the environmental sources and identified as *C. neoformans* var. *grubii* (serotype A) and var. *neoformans* (serotype AD/D). Amplification using the *LAC1* and *CAP64* primers resulted in the formation of DNA bands of unique size and pattern for each of the isolates, irrespective of molecular type, variety or serotype. The differences that exist between the serotype A and D isolates collected from various environmental sources had already been discussed by several researchers. [29, 30,32] Kwon-Chung and Bennett [32] found that 70% of cryptococcal isolates in Europe, Asia, North America, Argentina and Australia belonged to serotype A, while serotype D made up only 9% of cryptococcal isolates found in Europe.

PCR-fingerprinting pattern based on minisatellite (M13) or microsatellite $(GACA)_4$ specific sequences as single primers sequence in the PCR has grouped the environmental isolates of *C. neoformans* into 4 major molecular types namely VNI, VNII, VNIII and VNIV. The first two (VNI and VNII) belonged to *C. neoformans* var. *grubii* (serotype A), while VNIII was linked with serotype AD (hybrid) and VNIV belonged to *C. neoformans* var. *neoformans* (serotype D). [4,13] Since the differences were described in biology, advanced studies on the virulence, molecular epidemiology, structure and population of this pathogenic organism have been carried out globally. [2,4,12,13,33-36]

The intraspecies genetic heterogeneity *via* molecular type of *C. neoformans* strains was identified by RAPD analysis using specific primers. With those primers, 2 prime fingerprint patterns such as I and II were determined among the positive environmental isolates (32 samples) of *C. neoformans* collected from Chennai. Our experiment with modified methods of Meyer *et al* [14] expressed good reproducibility of the RAPD report. Our present study design also favoured the comparison of genetic relatedness among environmental isolates; it was clearly shown that droppings of pigeon contained genetically heterogeneous population of *C. neoformans*. We observed a high genetic variability among isolates of *C. neoformans* with the aid of primers, as demonstrated in previous studies. [37-39] Future studies about *C. neoformans* strains from clinical sources are necessary to determine the detailed genotypic and phenotypic characterisation, and hence attain a healthier understanding of cryptococcosis epidemiology distributed in Tamil Nadu, India.

CONCLUSION

The results obtained from the present investigation provide additional information to the existing knowledge on the molecular epidemiology of environmental *Cryptococcus neoformans* strains isolated from pigeon droppings in the selected regions of South India. Considering the increasing growth rate of immunocompromised patients in India, the possibility of opportunistic cryptococcal infections is also high. A thorough knowledge of the reservoir status of *C. neoformans* with a detailed molecular characterization study is needed in future for a better understanding of the epidemiology of cryptococcus related infections and its proper management in India.

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REFERENCES

1. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of America. *Clin Infect Dis* 2010; 50: 291 – 322.
2. Heiman J, Kozel TR, Kwon-Chung KJ, Perfect JR, Casadevall A. Cryptococcus from human pathogen to model yeast. *Washington, DC: ASM Press* 2011.
3. Velagapudi R, Hsueh YP, Geunes-Boyer S, Wright JR, Heitman J. Spores as infectious propagules of *Cryptococcus neoformans*. *Infect Immun* 2009; 77: 4345-55.
4. Boekhout T, Theelen B, Diaz M. Hybrid genotypes in pathogenic yeast *Cryptococcus neoformans*. *Microbiol* 2001; 147: 891 – 907.
5. Kwon-Chung KJ, Varma A. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Res* 2006; 6: 574 – 587.
6. Lengeler KB, Cox GM, Heitman J. Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. *Inf & Immun* 2001; 69:115 – 122.
7. Sasaki K, Matsusaka N, Sato I, Tsuda S, Tsutsumi K, Ito K, Hasegawa A, Pal M. Isolation of *Cryptococcus neoformans* from pigeon excreta. *J Japanese Vet Med Assoc* 1999; 52:521-524.
8. Zarrin M, Jorfi M, Amirrajab N, Rostami M. Isolation of Cryptococcus neoformans from pigeon droppings in Ahwaz, *Iran. Turk J Med Sci*. 2010; 40: 313-6.
9. Boekhout T, Van Belkum A, Leenders AC. Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. *Int J Systematic Bacteriol* 1997; 47: 432 – 442.
10. Garcia-Hermoso D, Janbon G, Dromer F. Epidemiological evidence for dormant *Cryptococcus neoformans* infection. *J Clin Microbiol* 1999; 37: 3204 – 3209.
11. Calvo BM, Colombo AL, Fischman O. Antifungal susceptibilities, varieties, and electrophoretic karyotypes of clinical isolates of *Cryptococcus neoformans* from Brazil, Chile, and Venezuela. *J Clin Microbiol* 2001; 39: 2348 – 2350.
12. Casali A, Goulart L, Rosa e Silva L, Ribeiro A, Amaral A, Alves S, Schrank A, Meyer W, Vainstein M. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Res* 2003; 3: 405 –415.
13. Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, Fisher M, Gilgado F, Hagen F, Kaocharoen S. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. *Med Mycol* 2009; 47: 561-70.
14. Meyer W, Castaneda A, Jackson S. Molecular typing of Ibero American Cryptococcus *neoformans* isolates. *Emerg Inf Dis J* 2003; 9: 189 – 195.
15. Trilles L, Lazera M, Wanke B, Theelen B, Boekout T. Genetic characterization of environmental isolates of *Cryptococcus neoformans* species complex from Brazil. *J Clin Microbiol* 2003; 41: 383 – 390.
16. Mseddi F, Sellami A, Jarboui MA, Sellami H, Makni F, Ayadi A. First environmental isolations of *Cryptococcus neoformans* and *Cryptococcus gatti* in Tunisia and review of published studies on

environmental isolations in Africa. *Mycopathologia* 2011; 171: 355-60.
17. Jang YH, Lee SJ, Lee JH, Chae HS, Kim SH, Choe NH. Prevalence of yeast-like fungi and evaluation of several virulence factors from feral pigeons in Seoul. *Korea Let App Microbiol* 2011; 53(4): 367–371
18. Delgado AC, Taguchi H, Mikami Y. Human cryptococcosis: relationship of environmental and clinical strains of *Cryptococcus neoformans* var. *neoformans* from urban and rural areas. *Mycopathologia* 2005; 1: 7–11.
19. Lee A, Toffaletti DL, Tenor J, Soderblom EJ, Thompson JW, Moseley MA. Survival defects of *Cryptococcus neoformans* mutants exposed to human cerebrospinal fluid result in attenuated virulence in an experimental model of meningitis. *Infect Immun* 2010; 78: 4213-25.
20. Kurtzman CP, Fell JW. The yeasts: a taxonomic study. 4. ed. Amsterdam, Elsevier Science 1998; 1055 p.
21. Kwon-Chung KJ, Edman JC, Wickes BL. Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Inf & Immun* 1992; 60: 602 – 605.
22. Ito-Kuwa S, Nakamura K, Aoki S, Vidotto V. Serotype identification of *Cryptococcus neoformans* by multiplex PCR. *Mycoses* 2007; 50:277–281.
23. Chaturvedi S, Rodeghier B, Fan J, McClelland CM, Wickes BL, Chaturvedi V. Direct PCR of *Cryptococcus neoformans* MATK and MATa pheromones to determine mating type, ploidy, and variety: a tool for epidemiological and molecular pathogenesis studies. *J Clin Microbiol* 2000; 38: 2007-2009.
24. Padhye AA, Chakrabarti A, Chander J, Kaufman L. *Cryptococcus neoformans* var. *gatii* in India. *J Med & Vet Mycol* 1993; 31:165-168.
25. Denning DW, Hope WW. Therapy for fungal diseases: opportunities and priorities. *Trends Microbiol* 2010; 18: 195-204.
26. Nagarajan S, Gugnani HC, Kowshik T. Case report. Meningitis due to *C. neoformans* var. *neoformans* serotype AD associated with pulmonary tuberculosis. *Mycoses* 2000; 43: 67-69.
27. Gokulshankar S, Ranganathan S, Ranjith MS, Ranjith Singh AJ. Prevalence, serotypes and mating patterns of *Cryptococcus neoformans* in the pellets of different avifauna in Madras, India. *Mycoses* 2004; 47: 310-314.
28. Girish Kumar CP, Prabu D, Hiroki Mitani, Yuzuru Mikami, Thangam Menon. Environmental isolation of *Cryptococcus neoformans* and *Cryptococcus gattii* from living trees in Guindy National Park, Chennai, South India. *Mycoses* 2009; 53: 262-264.
29. Cogliati M. Global molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii*: an atlas of the molecular types. *Scientifica (Cairo)* 2013; 675-213.
30. Bennett JE, Kwon-Chung KJ, Howard DH. Epidemiological differences among serotypes of *C. neoformans*. *American J Epidemiol* 1977; 105: 582-586.
31. Khayhan K, Hagen F, Pan W, Simwami S, Fisher MC,Wahyuningsih R, Chakrabarti A, Chowdhary A, Ikeda R, Taj-Aldeen SJ, et al. Geographically structured populations of Cryptococcus neoformans variety grubii in Asia correlate with HIV status and show a clonal population structure. *PLoS One* 2013; 8: 72222.
32. Kwon-Chung KJ, Bennett JE. Epidemiological differences between the two varieties of *Cryptococcus neoformans*. *American J Epidemiol* 1984; 120: 123-130.
33. Campbell LT, Fraser JA, Nichols CB, Dietrich FS, Carter D, Heitman J. Clinical and environmental isolates of *Cryptococcus gattii* from Australia that retain sexual fecundity. *Eukaryotic Cell* 2005; 4: 1410–1419.
34. Jain N, Wickes BL, Keller SM, Fu J, Casadevall A, Jain P, Ragan MA, Banerjee U, Fries BC. Molecular epidemiology of clinical *Cryptococcus neoformans* strains from India. *J Clin Microbiol* 2005; 43: 5733–5742.

35. Litvintseva AP, Thakur R, Reller LB, Mitchell TG. Prevalence of clinical isolates of *Cryptococcus gattii* serotype C among patients with AIDS in Sub-Saharan Africa. *The J Inf Dis* 2005; 192: 888–892.
36. Tay ST, Lim HC, Tajuddin TH, Rohani MY, Hamimah H, Thong KL. Determination of molecular types and genetic heterogeneity of *C. neoformans* and *C. gattii* in Malaysia. *Med Mycol* 2006; 44: 617–622.
37. Pedroso RS, Ferreira JC, Lavrador MA, Maffei CM, Candido RC. Evaluation of the experimental inoculation of *Cryptococcus albidus* and *Cryptococcus laurentii* in normal mice: virulence factors and molecular profile before and after animal passage. *Mycopathologia* 2009; 168(2):59–72.
38. Kaocharoen S, Ngamskulrungroj P, Firacative C, Trilles L, Piyabongkarn D, Banlunara W, Poonwan N, Chaiprasert A, Meyer W, Chindamporn A. Molecular epidemiology reveals genetic diversity amongst isolates of the Cryptococcus neoformans/C. gattii species complex in Thailand. *PLoS Negl Trop Dis* 2013; 7: 2297.
39. Takashima M, Sugita T, Shinoda T, Nakase T. Three new combinations from the *Cryptococcus laurentii* complex: *Cryptococcus aureus*, *Cryptococcus carnescens* and *Cryptococcus peneaus*. *Int J Systematic & Evolutionary* 2003; 53: 1187–1194.

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