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# *Cryptococcus neoformans* isolated from the excreta of avian species in zoological gardens of Gujarat state

**RA Mathakiya, RG Jani, A Roy and MK Jhala**

**Abstract**
*Cryptococcus neoformans* is an opportunistic yeast that causes life-threatening infections such as meningoencephalitis in human, animals and birds. The source of this organism is mainly pigeon excreta; however, other avian species' excreta are implicated as a source of this yeast. In the present study, a total of 607 avian droppings collected from four different zoos of Gujarat state and screened to know the prevalence of *C. neoformans*. Out of 607 avian droppings 33 *C. neoformans* isolates were recovered from zoo avian droppings. Zoo wise prevalence of *C. neoformans* from avian droppings was found to be 7.25 per cent (14/193) in Vadodara Zoo, 5.34 per cent (14/262) in Ahmedabad Zoo, 3.61 per cent (3/83) in Junagadh Zoo and 2.90 per cent (2/69) in Surat Zoo. Bird order wise prevalence of *C. neoformans* recorded was 12.70 per cent in order Pscittaciformes (24/189), followed by 8.20 per cent in Columbiformes (5/61), 8.00 per cent in Galliformes (2/25) and 3.85 per cent in Anseriformes (1/26). All the isolates showed cultural characters on different media *viz*. SDA with chloramphenicol, Sunflower seed agar and Bird seed agar were indicative of *Cryptococcus* spp. and were further examined and identified microscopically and biochemically, and biotyped using CGB agar. The nested PCR and CN4/CN5 PCR revealed that all 33 isolates were either *C. neoformans*or *C. gattii*. Molecular typing was done using *URA5*-RFLP which revealed RFLP pattern similar to WM 148 (serotype A, VNI/AFLP1). This confirmed that all 33 isolates were of *C. Neoformans* var *grubii* serotype A (VNI) and PCR based mating type detection revealed MATα strains in all the isolates.

**Keywords:** Avian droppings, *Cryptococcus neoformans*, Gujarat state, MATα strains, Nested PCR, Molecular typing, *URA5*-RFLP

## Introduction
Cryptococcosis is a fungal disease found worldwide in human and animal populations. The causative agent is the organism *Cryptococcus* spp. which is considered infectious only as a desiccated yeast cell or basidiospore as found in the environment. *Cryptococcus* spp. are environmental fungi of the phylum *Basidiomycota*, class *Heterobasidiomycetes*, order *Filobasidiales* and family *Filobasidiaceae*. The genus *Cryptococcus* includes over 39 species however, only *C. neoformans* is considered to be pathogenic. There were previously three recognized varieties of *Cryptococcus neoformans*: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gattii* (serotypes B and C) as well as a hybrid of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (serotype AD). Serotypes are recognised based on the antigenicity of the capsular polysaccharides. Recently proposed changes to the nomenclature suggest that *C. neoformans*should be divided into two distinct species including *C. neoformans* (serotypes A, D and AD) and *C. gattii* (serotypes B and C) based on molecular and mating type characteristics. They are grouped into eight major molecular types, VNI-VNIV for *C. neoformans* and VGI-VGIV for *C. gattii*. These major molecular types differ in their host range, epidemiology, antifungal susceptibility and geographic distribution [1].
The ecology of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* are quite similar. As *C. neoformans* var. *grubii* was only proposed as a distinct variety separate from *C. neoformans* var. *neoformans* in 1999, it is often difficult to discern differing distributions of the two varieties in the literature. Historically, both varieties of *C. neoformans* were thought to be associated with avian excreta, particularly that of pigeons [2].

## Materials and Methods
### Reference Fungal Strains
The standard strains of *C. neoformans* and *C. gattii* representing each molecular type were supplied by Dr. Wieland Meyer, Chief Scientist, Molecular Mycology Research Laboratory,

The University of Sydney, Sydney Medical School-Westmead, Australia. Following standard strains representing each molecular type were included in the analysis: WM 148 (serotype A, VNI/AFLP1), WM 626 (serotype A, VNII/AFLP1A), WM 628 (serotype AD, VNIII/AFLP2), WM 629 (serotype D, VNIV/AFLP3), WM 179 (serotype B, VGI/AFLP4), WM 178 (serotype B, VGII/AFLP6), WM 175 (serotype B, VGIII/AFLP5), and WM 779 (serotype C, VGIV/AFLP7).

**Collection of bird droppings and other samples**

Fresh as well as desiccated excreta samples of birds were collected from surrounding of pigeon dwelling area and from captive birds like Anseriformes (ducks, geese and swans), Casuariiformes (emus and cassowary), Ciconiiformes (egrets, flamingos, herons, ibises, spoonbills and storks), Columbiformes (doves and pigeons), Falconiformes (vultures), Galliformes (chickens, peafowl/peacock, pheasant and turkeys), Gruiformes (cranes/kunj), Passeriformes (crows, finches, mynahs and sparrows), Peliconiformes (pelicans), Piciformes (hornbills), Pscittaciformes (budgerigar, cockatiels, cockatoos, lories, love birds, macaws, parakeets and parrots) and Strigiformes (owls) of different zoos of Gujarat state *viz*. Kamla Nehru Zoological Garden, Ahmedabad; Sayajibaug Zoo, Vadodara; Sarthana Zoo, Surat and Shakkarbaugh Zoo, Junagadh. The birds dropping samples were collected using sterile wooden spatulas and were transferred to sterile plastic bags. The plastic bags were labelled properly according to species of bird, name of zoo and date. Then, the samples were carried to the laboratory under refrigeration and stored until used.

**Sample Processing**

The portion of excreta (approximately 1-2 g) from each sterile plastic bag was aseptically removed, weighed, and transferred to test tube containing 9 ml saline solution (0.9%). The material was homogenized by shaking and allowed to stand for 30 minutes. The supernatant was aliquoted and streaked onto Sabouraud dextrose agar medium with chloramphenicol (SDA w/C), Bird seed agar (BSA) and Sunflower seed agar (SSA) using sterile cotton swab. The inoculated plates were incubated at 37 ℃ and were observed daily for 7 to 10 days.

Cultural isolation and identification of *Cryptococcus neoformans*

**Colony characters and microscopic examination**

BSA was prepared by the formulation of Staib's medium (Hi Media Ltd, Mumbai, India) for the identification of *C. neoformans*. This organism has phenoloxidase activity located within the cell wall and is able to metabolize caffeic acid. *Guizotia abysinica* seeds (Niger seeds) serve as the substrate for phenoloxidase. *C. neoformans* produces an enzyme phenoloxidase in which the substrate will be converted to melanin or a melanin-like pigment as the organism metabolizes *o*- and *p*-diphenols, resulting in dark brown color pigmented colonies. A positive reaction results in the production of a dark-brown pigment in the agar within 7 days of incubation at 37 ℃ temperature. Cryptococci other than *C. neoformans* or *C. gattii* do not produce the brown pigment on Niger seed agar.

On BSA and SSA light to dark brown pigmented colonies of *C. neoformans* were seen within 7 to 10 days at 37 ℃. The colonies selectively absorb a brown pigment from this medium and produces light to dark brown pigmented colonies. On SDA w/C, *C. neoformans* grew rapidly and developed moist, soft, glistening to dull, smooth, usually mucoid to pale yellow colonies within 3 to 5 days at 37 ℃ and were observed daily for 7 to 10 days.

*C. neoformans* shows spherical shaped yeast cells with or without budding and surrounded by different size of capsule in India ink preparation and on Gram staining Gram positive oval shaped yeast cells with or without budding.

**Biochemical characterization and Biotyping of *Cryptococcus* spp. isolates**

The biochemical characterization of *C. neoformans* isolates was done using urease test, nitrate reduction test, cycloheximide (0.1%) sensitivity test, sugar fermentation test.

Biotyping of the isolates was done according using L-Canavanine Glycine Bromothymolblue (CGB) agar growth test for differentiation of *C. neoformans* and *C. gattii*. CGB is the medium of choice to differentiate *C. gattii* from *C. neoformans*. This simple serotype or biotype test is based on the ability of *C. gattii* isolates to grow in the presence of L-canavanine and to assimilate glycine as a sole carbon source. *C. neoformans* is sensitive to L-canavanine which can not grow in the presence of L-canavanine.

CGB agar plates were prepared and were inoculated by using a loop to streak the organism down the center of the plate and then in a side-to-side motion. Reactions on CGB agar were considered positive if color changes from light yellow-green to cobalt blue produced during 48-72 hrs of incubation at 37 ℃ temperature indicating *C. gattii* (serotype B/C) and negative if the color of the CGB medium remained yellow indicating the var. *neoformans* (serotype A/D). A residual light-blue color around the inoculum was interpreted as a negative reaction.

**PCR based detection of *Cryptococcus* spp. isolates**

***Template DNA preparation***

The standard strains of *C. neoformans* as well as *C. gattii* and all the *Cryptococcus* isolates were grown for 48 to 72 hrs in BHI broth at 37ºC. The obtained culture (approximately 0.5 ml) was centrifuged at 4ºC in a cooling microcentrifuge (Sigma, USA) at 6000 revolutions per minute (rpm) for 10 min. The recovered pellet was resuspended in 100 µl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA). The samples were heated at 95ºC for 15 min, cell debris was removed by centrifugation and 3 µl of the supernatant was used as a DNA template in PCR reaction mixture.

***Identification of Cryptococcus spp. isolates by nested PCR***

The nested PCR was done according to methodology described by Bialek *et al*. [3] with suitable modifications. The nested PCR was done using oligonucleotide primers Fungus I (5'-GTTAAAAAGCTCGTAGTTG-3') and Fungus II (5'-TCCCTAGTCGGCATAGTTTA-3') which were complementary to highly conserved regions within the nuclear gene coding for a small subunit of rRNA (18S rDNA) of several pathogenic fungi, including *C. neoformans* and *C. gattii*, and generated a 429 bp amplicon. PCR were carried out in a mixture of 25 µl containing DNase-RNase free water-7.50 µl, 2X PCR master mix-12.50 µl, Forward Primer (10 pmole/µl)-1.00 µl, Reverse Primer (10 pmole/µl)-1.00 µl, DNA Template-3.00 µl. PCR protocol was initial denaturation at 94ºC for 5 min was followed by 35 cycles at 94ºC for 30 sec, annealing at 50ºC for 30 sec and elongation at 72ºC for 1

min, and a final elongationat 72ºC for 5 min. The oligonucleotides Cryp I (5'-TCCTCACGGAGTGCACTGTCTTG-3') and Cryp II (5'-CAGTTGTTGGTCTTCCGTCAATCTA-3') were complementary to *C. neoformans* and *C. gattii* selective regions within the 18S rDNA target, spanning a 278 bp region, and served as nested primers for the Fungus I/II amplicon. The PCR protocol was initial denaturation at 94ºC for 5 min was followed by 35 cycles at 94ºC for 45 sec, annealing at 50ºC for 45 sec and elongation at 72ºC for 1 min, and a final elongation at 72ºC for 10 min.

The PCR was done using CN4 and CN5 primers according to Mitchell *et al.* [4] with suitable modifications. The primers CN4 (5'-ATCACCTTCCCACTAACACATT-3') and CN5 (5'-GAAGGGCATGCCTGTTTGAGAG-3') amplify specific segment of the genes coding for rRNA (rDNA) of *C. neoformans* and *C. gattii*. These primers were on the basis of a comparative analysis of sequences of the 5.8S and internal transcribed spacer (ITS) regions of *Filobasidiella neoformans* and *Filobasidiella bacillispora*, which are the teleomorphs of *C. neoformans* and *C. gattii*, respectively.

*Identification of mating type of Cryptococcus spp. from the isolates by PCR amplification of STE12α gene*

The mating type detection of *Cryptococcus* spp. isolate was done according to Bovers *et al.* [5]. Primer STE12αF809 (5'-TTGACCTTTTRTTCCGCAATG-3') STE12αR1607 (5'-TTTCTTCTCCCCTGTTTATAGGC-3') amplifies a 760 bp region specific for both *C. neoformans* and *C. gattii MAT*α strains.

*Visualization of PCR products by agarose gel electrophoresis*

To confirm the targeted PCR amplification, 5 µl of the PCR products from each tube was mixed with 1 µl of 6X gel loading buffer and electrophoresed along with DNA molecular weight marker (Gene Ruler, MBI Fermentas) on 2.0% agarose gel containing ethidium bromide (at the rate of 0.5 µg/ml) at 5V/cm for 60 min in 0.5 X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (SynGene, Gene Genius BioImaging System, UK).

**PCR-RFLP Analysis**

All the isolates along with reference strains mentioned earlier were used for PCR-RFLP analysis. The molecular typing of *Cryptococcus* spp. isolates was done using *URA5*-RFLP according to Meyer *et al.* [6] using primers URA5(5'-ATGTCCTCCCAAGCCCTCGACTCCG-3') and SJ01 (5'-TTAAGACCTCTGAACACCGTACTC-3'). The total 30µl reaction mixture for RE digestion was prepared in a 200 µl PCR tube which includes Nuclease free water-15.0µl, 10X buffer-3.0µl, Restriction enzymes (10U/µl): Sau96I (FD, MBI Fermentas)-1.0µl, HhaI (FD, MBI Fermentas)-1.0 µl, PCR product-10.0µland incubated for 45 min at 37 ℃.

After restriction digestion, the PCR products of *URA5* gene fragments were electrophoresed on 3% agarose gel (according to the expected size of fragments) containing 1% ethidium bromide @ 5 µl/100ml by submarine gel electrophoresis apparatus at constant voltage of 80V for 3 hrs using 0.5X TBE buffer. The loading dye used was Bromophenol blue @ 2.0 µl per 10 µl of RFLP product. Mass Ruler Low range DNA Ladder (Range, 100-1500 bp) was used as a molecular size marker. The bands were visualized under UV light and documented by gel documentation system. The band size were judged by comparing with molecular size marker and recorded.

**Results**

Zoo wise prevalence of *C. neoformans*

A total of 607 avian droppings collected from four different zoos of Gujarat state were screened to know the prevalence of *C. neoformans*. Zoo wise prevalence of *C. neoformans* from avian droppings was found to be 7.25 per cent (14/193) in Vadodara Zoo, 5.34 per cent (14/262) in Ahmedabad Zoo, 3.61 per cent (3/83) in Junagadh Zoo and 2.90 per cent (2/69) in Surat Zoo. None of the other samples except one each of soil and feather and nodular swab was positive for *C. neoformans*.

**Bird order wise prevalence of *Cryptococcus* spp.**

In the present study, order wise prevalence of *C. neoformans* was recorded and the highest prevalence was observed in birds classified under the order Pscittaciformes (12.70%), which includes budgerigar, cockatiels, cockatoos, lories, love birds, macaws, parakeets, rosella; followed by Columbiformes (8.20%), which includes pigeons; Galliformes (8.00%), which includes pheasants; and Anseriformes (3.85%), which includes ducks. The prevalence of Gruiformes (10%), which includes kunj, was not considered due to very small sample size (n=10) (Table 1). *C. neoformans* could not be isolated from the birds of orders Casuariiformes (emus and cassowary), Ciconiiformes (egrets, flamingos, herons, ibises, spoonbills and storks), Falconiformes (vultures), Passeriformes (crows, finches, mynahs and sparrows), Peliconiformes (pelicans), Piciformes (hornbills) and Strigiformes (owls).

**Cultural isolation and identification**

A total of 104 fungal isolates were recovered from 607 samples. Of these isolates, 33 isolates showed cultural characters on different media indicative of *Cryptococcus* spp. Other non-*Cryptococcus* spp. and other fungal isolates were not further processed. All 33 isolates of *C. neoformans* showed the colony characters on different media *viz*. Sabouraud dextrose agar medium with chloramphenicol, Sunflower seed agar medium, Bird seed agar, and Brain heart infusion (BHI) agar. All 33 *C. neoformans* isolates showed spherical shaped yeast cells with or without budding surrounded by variable size of capsule in India ink preparation. On Gram staining, Gram positive oval shaped yeast cells with or without budding were observed.

**Biochemical characterization of the isolates**

Out of 33 *C. neoformans* isolates, 24 (72.73%) isolates showed strong urea hydrolysis on Christensen urea agar as indicated by dark pink colouration of media, where as 9 (27.27%) isolates were weak positive for urea hydrolysis as indicated by light pink colouration of media. All 33 *C. neoformans* isolates were negative for nitrate reduction, and sensitive to cycloheximide.

All 33 *C. neoformans* isolates revealed a similar pattern for sugar utilization *viz*. positive for glucose (G), galactose (Ga), sucrose (Su), trehalose (Te), maltose (Ma), rhamnose (Rh), D-xylose (Xy),inositol (Is), mannitol (Mn), arabinose (Ar) and sorbitol (Sb). All the isolates were negative for lactose (La) and melibiose (Mb) utilization. Variable sugar fermentation reactions were observed with raffinose (Rf) and cellobiose (Ce). Out of 33 *C. neoformans* isolates, 19 (57.58%) isolates showed raffinose (Rf) positive and 14 (42.42%) isolates

showed cellobiose (Ce) positive reactions.

**Biotyping of *Cryptococcus* spp. isolates**

All the 33 isolates of *Cryptococcus* spp. on CGB agar showed no colour change of yellow coloured CGB medium indicating that all the isolates were *C. neoformans* i.e. (serotype A or D).

PCR based detection of *Cryptococcus* spp. isolates

The nested PCR was done using oligonucleotide primers Fungus I and Fungus II which were complementary to highly conserved regions within the nuclear gene coding for a small subunit of rRNA (18S rDNA) of several pathogenic fungi, including *C. neoformans* and *C. gattii*, and generated a 429 bp amplicons. All 33 isolates of *C. neoformans* generated 429 bpamplicons indicating them to be fungal isolates. The oligonucleotides Cryp I and Cryp II were complementary to *C. neoformans* and *C. gattii* selective regions within the 18S rDNA target, spanning a 278 bp region, and served as nested primers for the Fungus I/II amplicon. All 33 *C. neoformans* isolates generated expected product of 278 bp with these primers indicating them to be either *C. neoformans*or *C. gattii*.

All 33 *C. neoformans* isolates were processed for PCR using CN4 and CN5 primers. All 33 *C. neoformans* isolates generated expected products of 136 bp indicating them to be either *C. neoformans* or *C. gattii*.

The mating type detection of all 33 *C. neoformans* isolates was done according to Bovers *et al.* [5]. Primers STE12αF809/STE12αR1607 amplified a 760 bp region specific for both *C. neoformans* and *C. gattii* MATα strains. All 33 *C. neoformans* isolates generated expected product of 760 bp indicating them to be *MAT*α strains.

***URA5*-RFLP analysis**

The molecular typing of all 33 *C. neoformans* isolates was done by PCR-RFLP using fast digest restriction enzymes *viz*. *Sau*96I and *Hha*I for digestion of PCR products after amplification of *URA5* gene of *C. neoformans.* The RFLP profile of all 33 *C. neoformans* isolates were compared with standard strains of *Cryptococcus neoformans* and *Cryptococcus gattii* representing each molecular type. All 33 *C. neoformans* isolates revealed RFLP pattern similar to WM 148 (serotype A, VNI/AFLP1). This confirmed that all 33 *C. neoformans* isolates were of *C. neoformans* var *grubii* serotype A (VNI) (Plate 1).

**Discussion**

*Cryptococcus neoformans* is primarily associated with nests and soils containing avian droppings, especially those of pigeons [7]. Despite the fact that both *Cryptococcus* species, *C. neoformans* and *C. gattii*, are capable of growth on pigeon guano, only *C. neoformans* exhibit prolific mating, completing its life cycle. Bird guano may represent the ecological niche for *C. neoformans* [8]. Studies reported that captive birds can promote the contamination of dwellings and public areas by *Cryptococcus* species [9]. Many reports have been published describing the development of cryptococcosis in people after exposure to birds or avian excreta, illustrating that human infection can result from these sources [10]. Therefore, avian species, especially the captive zoo birds, have the potential to disseminate *C. neoformans* and other zoonotic agents because they are popular as pets.

Domestic and wild birds are known to be possible carriers of fungi that are pathogenic to humans and other animals [11]. In particular, several studies have been carried out on the occurrence of *C. neoformans* in bird droppings in psittaciformes [12], passeriformes, columbiformes [13] and falconiformes [14]. *C. neoformans* and *C. gattii* can cause cryptococcosis in humans as well as in both wild and domestic animals [15].

**Prevalence of *Cryptococcus* spp. by cultural isolation and identification**

In the present study, out of total 607 samples screened, 5.44 per cent (33/607) samples were found to be positive for *C. neoformans* (cent per cent *C. neoformans* var. *grubii*, serotype A). Steenbergen and Casadevall (2000), who analysed 40 *C. neoformans* isolates and found that 39 were typeable, of which 85% and 12.5% were *C. neoformans*var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D), respectively. Casali *et al.* [7] studied 105 clinical and 19 environmental (pigeon excreta and *Eucalyptus* spp.) isolates and found that majority of the clinical and environmental isolates analyzed belonged to *C. neoformans* var. *grubii* serotype A (89.5 and 52.6%, respectively). Nishikawa *et al.* [16] reported the greater frequency of serotype A in pigeon samples. They observed that there is worldwide association of *C. neoformans* serotype A with cryptococcosis, among HIV-positive individuals. Abegg *et al.* [9] studied environmental isolates of *C. neoformans* by analyzing fecal samples from 10 (18.18%) out of 59 species of captive birds and reported that 33 isolates (87%) were *C. neoformans* var. *grubii*. Baroni *et al.* [17] studied 576 environmental isolates and revealed that 98.8% of the strains were serotype A (var. *grubii)* and just 1.2% serotype AD (var. *neoformans*). Costa *et al.* [18] collected 11 environmental samples (4 caged psittacine bird's dried excreta and 7 decaying wood material) and 2 samples were identified as *C. neoformans* serotype A. Liaw *et al.* [19] tested 100 cryptococcal isolates and reported that 99 isolates including all 8 pigeon isolates were serotype A.

Andrade-Silva *et al.* [20] studied 73 samples comprising of 62 from bird droppings and 11 from tree detritus. They isolated *C. neoformans* alone in 43.8%, *C. laurentii* alone in 23.3% and both fungi together in 10.9% samples.

Ferreira-Paim *et al.* [21] studied 253 samples of bird excreta i.e. 120 fresh and 133 dry excreta from pet shop cages and houses in different neighbour hoods. They isolated *C. neoformans* in 19 (14.28%) dry samples and one fresh sample (0.84%). The result of this study indicated that dry bird excreta possess more number of *C. neoformans* due to acidification of excreta which promotes the growth of fungus. In present study, fresh droppings were more as compared to dry one, probably resulting in poor isolation rate (5.80%). Gonzalez-Hein *et al.* [22] recovered *C. neoformans* from 17 of the 113 samples (15%). In contrast to present study, Pal *et al.* [23] could not demonstrate *C. neoformans* in 107 samples of avian droppings in Japan. Kielstein *et al.* [24] also did not isolate any *C. neoformans* strains from the 30 samples of pigeon excreta. Absence of *C. neoformans* in fecal specimens from psittacines was also found by Mancianti *et al.* [25].

**Zoo wise prevalence of *Cryptococcus* spp.**

In the present study, a total of 607 avian droppings collected from four different zoos of Gujarat state were screened to know the prevalence of *C. neoformans*. Zoo wise prevalence of *C. neoformans* from avian droppings was found to be highest i.e. 7.25 per cent (14/193) in Vadodara Zoo, followed by 5.34 per cent (14/262) in Ahmedabad Zoo, 3.61 per cent (3/83) in Junagadh Zoo and 2.90 per cent (2/69) in Surat Zoo.

Location wise difference in prevalence of *C. neoformans* has been reported [26]. Although it is difficult to explain the difference in prevalence at 4 different zoos observed in present study, it might be logical to assume that the samples collected from Vadodara zoo were drier and also the free living pigeons in Vadodara and Ahmedabad zoo areas were more than the other zoos. Dry avian excreta and soils contaminated with such droppings have been reported to contain more *C. neoformans* than moist excreta [7, 27]. Manciantie*t al.* [25] could not find *C. neoformans* or *C. gattii* in 325 samples of fresh excreta of Psittaciformes.

**Bird order wise prevalence of *Cryptococcus* spp.**

In the present study, order wise prevalence of *C. neoformans* was recorded and the highest prevalence was observed in birds classified under the order Pscittaciformes (12.70%), which includes budgerigar, cockatiels, cockatoos, lories, love birds, macaws, parakeets, rosella; followed by Columbiformes (8.20%), which includes pigeons; Galliformes (8.00%), which includes pheasants; and Anseriformes (3.85%), which includes ducks. The prevalence of Gruiformes (10%), which includes kunj, was not considered due to very small sample size (n=10). *C. neoformans* could not be isolated from the birds of orders Casuariiformes (emus and cassowary), Ciconiiformes (egrets, flamingos, herons, ibises, spoonbills and storks), Falconiformes (vultures), Passeriformes (crows, finches, mynahs and sparrows), Peliconiformes (pelicans), Piciformes (hornbills) and Strigiformes (owls).

Lugarini *et al.* [28] isolated *C. neoformans* from 34 (24.11%) Passerine samples and two (1.42%) Psittacine samples, with an overall occurrence of 25.53%. The prevalence in Pscittacine samples recorded by them was very low as compared to our findings. Despite the fact that pigeon droppings are commonly documented as the major source of *C. neoformans* in nature, the yeast has also been isolated in high rate from droppings of caged birds including both Passerine and Psittacine birds [29]. *C. neoformans* is generally isolated from avian droppings, whereas *C. gattii* is not typically isolated [8].

*C. neoformans* has been isolated from various sources in nature and is commonly associated with pigeon (order Columbiformes) droppings and less frequently with the droppings of other birds, such as order Psittaciformes and Passeriformes [14, 30]. In the present study, *C. neoformans* was isolated not only from Columbiformes (8.20%) but also from Pscittaciformes (12.70%), Gruiformes (10%), Galliformes (8%), and Anseriformes (3.85%), while none of the isolate was recovered from Passeriformes.

The high prevalence of *C. neoformans* in Pscittaciformes (12.70%) and Columbiformes (8.20%) appears valid as the sample size were sufficient, however high prevalence recorded in Gruiformes (10%) can not be considered valid as the sample size was less (n=10). The mechanism through which the particular bird's excreta become infected is still uncertain [30, 31]. However, the development of *C. neoformans*in birds' excreta can be attributed to the large quantity of fungic cells in soil or air, dispersed by wind, which find a rich environment in excreta for their proliferation. It is widely known that non-infected pigeon excreta become infected when exposed to air containing aerosolubilized cells of *C. neoformans*.

**PCR based detection of *Cryptococcus* spp. isolates**

The nested PCR of all 33 isolates of *C. neoformans* generated expected product of 278 bp with these primers indicating them to be either *C. neoformans* or *C. gattii*. All 33 *C. neoformans* isolates were also processed for PCR using CN4 and CN5 primers which generated expected product of 136 bp indicating them to be either *C. neoformans* or *C. gattii*. The primers CN4 and CN5 amplify specific segment of the genes coding for rRNA (rDNA) of *C. neoformans* and *C. gattii* [4]. The mating type detection of all 33 *C. neoformans* isolates generated the expected product of 760 bp confirming them to be of MATα strains. Knowledge of mating type is important for understanding the ecology and virulence of this fungus [32]. Both *C. neoformans* and *C. gattii* are haploid and heterothallic and have a bipolar mating system with single mating type loci and two alleles: *MATα* and *MAT*a. The MATα strains have been shown to be more prevalent in clinical and environmental isolates and also more virulent than MATa [33]. Our result endorses the finding of Casali *et al.* [34], who studied 105 clinical and 19 environmental samples and found mating type "α" in 98.1 and 94.7 per cent pigeon excreta and *Eucalyptus* spp. samples, respectively. Tay *et al*. [35] studied 544 samples for the occurrence of *C. neoformans* in bird excreta in Klang valley, Malaysia. They isolated 20 strains of *C. neoformans* var *grubii* (serotype A) out of 544 samples of bird excreta collected from a local zoo, pet shops and public areas, and reported that all the strains were of "α" mating types, as determined by a pheromone specific PCR assay. Their result supports the present study in which 100% isolates were of mating type "α". Chen *et al.* [36] analyzed the genotype of 120 *C. neoformans* and 9 *C. gattii* strains, and found 120 of the 129 isolates as *C. neoformans* serotype A, mating type MATα strains.

There were several interesting observations implicating mating type as a virulence factor. Firstly, MATα cells were much more prevalent than MATa cells. For instance, in a survey of natural and clinical isolates, the MATα mating type was 40-fold more abundant in environmental isolates and 30-fold more abundant in clinical isolates than its MATa counterpart [33]. In addition, most of the Vancouver isolates were "α" mating type [37]. Secondly, when congenic "α" and "a" strains (JEC21) of serotype D (genetically identical except at the mating type locus) were studied in a murine model of cryptococcosis, the MATα strain was found to be significantly more virulent than the MATa strain [38]. Congenic "α" and "a" cells in the serotype A H99 showed the same pathogenicity level in various mammalian models [39], but "α" cells have an enhanced predilection to penetrate the CNS during coinfection with "a" cells, which provides an explanation for the prevalence of "α" strains in clinical isolates [40]. Therefore, it is logical to assume that all the 33 isolates obtained in the present study can behave as more virulent *C. neoformans* and may turn into potential pathogens upon favourable conditions.

**Biotyping and molecular characterization of *Cryptococcus* spp. isolates**

All the 33 isolates of *Cryptococcus* spp. on CGB agar showed that all isolates were *C. neoformans* i.e. (serotype A/D). *Cryptococcus gattii* grows in the presence of L-canavanine and utilizes glycine as the sole carbon and nitrogen source, which raises the pH of the medium and results in blue color of the medium by bromothymol blue indicator. *Cryptococcus neoformans* will not grow in the presence of canavanine and does not utilize glycine, so the CGB medium color remains unchanged [41]. Identification of pathogenic *Cryptococcus*

species and subspecies are important both for clinical management as well as for epidemiology. False-positive *C. gattii* results have been reported using the conventional CGB differentiation method due to the existence of *C. neoformans* isolates that are resistant to canavanine [42]. Meanwhile, PCR fingerprinting and PCR-RFLP are being utilized more frequently [43]. PCR-RFLP was therefore applied in the present study to confirm the serotypes of the *Cryptococcus* isolates, and to ensure the results obtained by CGB medium cultivation. In the current study, PCR-RFLP i.e. *URA5*-RFLP was used to characterize the *Cryptococcus* isolates and all were found to be of molecular type VNI. This result was consistent with previous studies that identified *C. neoformans* VNI as the primary agent of cryptococcosis [16, 44]. A study that characterized 63 Brazilian isolates described the prevalence of the molecular types VNI (82.3%), VGII (13.6%) and VNII (3.0%). Studies in the Pará state of Brazil reinforced the prevalence of VNI and VGII as being the principal molecular types of *C. neoformans* and *C. gattii*, respectively, in the northern region of Brazil [6].

Ribeiro and Ngamskulrungroj [45] collected 33 samples of pigeon excreta and performed PCR amplification with primer M13 and *URA5*-RFLP analysis. They found that all the isolates were serotype A (*C. neoformans* var. *grubii*), genotype VNI, MATa mating type, but only 50% were able to mate *in vitro* with the opposite mating type MATαtester strains (JEC20, KN99a and Bt63). Liaw *et al.* [19] investigated 100 clinical isolates, among which 99 were *C. neoformans* var. *grubii* serotype A and one was *C. gattii* serotype B. All of these isolates were α mating type. PCR fingerprinting generated by primers M13 and $(GACA)_4$, and *URA5*-RFLP analysis revealed that *C. neoformans* var. *grubii* isolates belonged to the VNI (98 isolates) and the VNII (one isolate) types, and the single *C. gattii* was VGI type.

Unifying the overall findings of the present study, it can be opined that the study has attempted apparently for the first time in India to know the prevalence of *C. neoformans* at potential sources especially the captive birds at zoos. Thus, the beginning made should go a long way to further strengthen our research work on this neglected but potential pathogen of animals, birds and humans. The study also standardized and applied many cultural, biochemical and molecular methods not only to identify *C. neoformans*, but also to characterize them at molecular level. The use of CGB medium along with the molecular typing using *URA5*-RFLP gives idea about variety and molecular types as well as serotypes of the isolates which is important for molecular epidemiology. The information generated should form a base in future to carry forward our understanding on cryptococcosis.

**Table 1:** Prevalence and identification of *C. neoformans* in different samples

| Sr. No. | Bird order | Avian species | Sample code | Identification | Molecular type | Mating type |
|---|---|---|---|---|---|---|
| 1 | Pscittaciformes (budgerigar, cockatiels, cockatoos, lories, love birds, macaws, parakeets, rosella) | Budgerigar | AZ-2 | *C. neoformans* var. *grubii* | VNI | MATα |
| 2 | | Chattering lory | AZ-12 | *C. neoformans* var. *grubii* | VNI | MATα |
| 3 | | Alexandrine parakeet | AZ-14 | *C. neoformans* var. *grubii* | VNI | MATα |
| 4 | | Cockatiel | AZ-18 | *C. neoformans* var. *grubii* | VNI | MATα |
| 5 | | Cockatoo | AZ-30 | *C. neoformans* var. *grubii* | VNI | MATα |
| 6 | | Rose breasted parakeet | AZ-64 | *C. neoformans* var. *grubii* | VNI | MATα |
| 7 | | Cockatoo | AZ-70 | *C. neoformans* var. *grubii* | VNI | MATα |
| 8 | | Cockatiel | AZ-90 | *C. neoformans* var. *grubii* | VNI | MATα |
| 9 | | Cockatiel | AZ-109 | *C. neoformans* var. *grubii* | VNI | MATα |
| 10 | | Rose breasted parakeet | AZ-118 | *C. neoformans* var. *grubii* | VNI | MATα |
| 11 | | Rose ringed parakeet | AZ-123 | *C. neoformans* var. *grubii* | VNI | MATα |
| 12 | | Crimson rosella | BZ-10 | *C. neoformans* var. *grubii* | VNI | MATα |
| 13 | | Blossom headed parakeet | BZ-14 | *C. neoformans* var. *grubii* | VNI | MATα |
| 14 | | Budgerigar | BZ-21 | *C. neoformans* var. *grubii* | VNI | MATα |
| 15 | | Golden cherry love bird | BZ-33 | *C. neoformans* var. *grubii* | VNI | MATα |
| 16 | | Alexandrine parakeet | BZ-53 | *C. neoformans* var. *grubii* | VNI | MATα |
| 17 | | Cockatiel white | BZ-63 | *C. neoformans* var. *grubii* | VNI | MATα |
| 18 | | Blue and golden macaw | BZ-68 | *C. neoformans* var. *grubii* | VNI | MATα |
| 19 | | Illegar macaw | BZ-88 | *C. neoformans* var. *grubii* | VNI | MATα |
| 20 | | Alexandrine parakeet | BZ-121 | *C. neoformans* var. *grubii* | VNI | MATα |
| 21 | | Cockatiel white | BZ-138 | *C. neoformans* var. *grubii* | VNI | MATα |
| 22 | | Cockatiel | BZ-206 | *C. neoformans* var. *grubii* | VNI | MATα |
| 23 | | Love bird | JZ-19 | *C. neoformans* var. *grubii* | VNI | MATα |
| 24 | | Budgerigar | JZ-76 | *C. neoformans* var. *grubii* | VNI | MATα |
| 25 | Columbiformes (pigeons) | Pigeon | AZ-26 | *C. neoformans* var. *grubii* | VNI | MATα |
| 26 | | Nicobar pigeon | AZ-42 | *C. neoformans* var. *grubii* | VNI | MATα |
| 27 | | Pigeon | AZ-80 | *C. neoformans* var. *grubii* | VNI | MATα |
| 28 | | White Jacobin pigeon | BZ-168 | *C. neoformans* var. *grubii* | VNI | MATα |
| 29 | | Brown Jacobin pigeon | BZ-187 | *C. neoformans* var. *grubii* | VNI | MATα |
| 30 | Galliformes (pheasants) | Silver pheasant | BZ-189 | *C. neoformans* var. *grubii* | VNI | MATα |
| 31 | | White pheasant | JZ-52 | *C. neoformans* var. *grubii* | VNI | MATα |
| 32 | Anseriformes (ducks) | Duck | SZ-65 | *C. neoformans* var. *grubii* | VNI | MATα |
| 33 | Gruiformes (kunj) | Kunj | SZ-53 | *C. neoformans* var. *grubii* | VNI | MATα |

AZ = Ahmedabad Zoo, BZ = Baroda Zoo, JZ = Junagadh Zoo, SZ = Surat Zoo

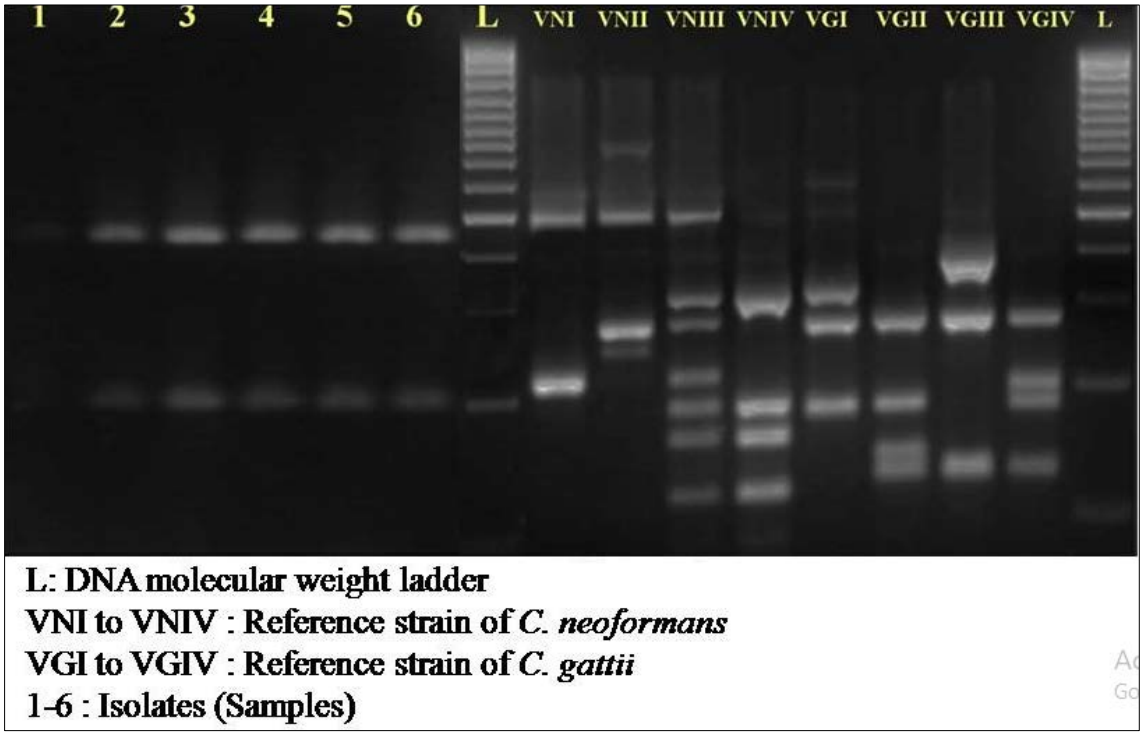


**Plate l:** *URA* 5-RFLP profile of Cryptococcus neoformans obtained by double digestion of *URA* 5 gene amplicon with *Sau*96I and *Hha*l

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