Central nervous system infection due to Cryptococcus gattii sensu lato in India: Analysis of clinical features, molecular profile and antifungal susceptibility

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Summary
Cryptococcus gattii species complex has evolved as a pathogen in the last two decades causing infection among both immunocompetent and immunocompromised hosts. We aimed to analyse the clinical features of CNS infection caused by C. gattii sensu lato, molecular and antifungal susceptibility profile of this pathogen. Cases diagnosed to have CNS cryptococcosis were included in the study. Cryptococcus recovered from patient’s specimen was identified by standard protocol. Species confirmation, mating type and molecular type determination were performed by PCR based methods. Antifungal susceptibility was tested in VITEK2C to amphotericin B, 5-flucytosine, fluconazole and voriconazole. Among 199 cases, 20 (10%) were due to C. gattii, comprising of 75% cryptococcal meningitis and 25% cryptococcoma cases. Young adult males were commonly affected. Headache and vomiting were prominent symptoms and 50% were immunocompromised. Among the isolates, 75%, 20% and 5% were C. tetragattii, C. gattii sensu stricto and C. bacillisporus respectively and all had mating type α. Four (20%) isolates of C. tetragattii and the only isolate of C. bacillisporus were resistant to fluconazole. The most common species isolated from South India is C. tetragattii. The study contributes to the epidemiology of C. gattii and reiterates the need for genotyping and antifungal susceptibility testing.

KEYWORDS
antifungal susceptibility, CNS infection, Cryptococcus gattii sensu lato, epidemiology, molecular profile

1 | INTRODUCTION

Cryptococcus neoformans, a fungus which belongs to phylum Basidiomycota, causes life-threatening infection in humans and animals. There are two main pathogenic species within the genus Cryptococcus: namely C. neoformans sensu lato (s.l.) and C. gattii sensu lato (s.l.). Currently, C. neoformans s.l. is recognized as a species complex comprising C. neoformans molecular type VNI/AFLP1, VNII/AFLP1B, VNB/AFLP1A, VNIII/AFLP3 (serotype A) and C. deneoformans VNI/AFLP2 (serotype D).1 Cryptococcus gattii s.l. (serotypes B and C) is recognized as a species complex distinct from C. neoformans s.l. due to their differences in electrophoretic karyotypes, DNA fingerprints, intergenic spacer sequences of the ribosomal DNA, and biochemical characteristics.1 Presently C. gattii s.l. includes five distinct species based upon numerous characteristics as described by Hagen et al.1; Cryptococcus gattii sensu stricto (s.s.) AFLP4/VG1, Cryptococcus bacillisporus AFLP5/VGII, Cryptococcus deuterogatii AFLP6/VGII, Cryptococcus tetragattii AFLP7/VGIV and Cryptococcus decagattii AFLP10 VGIV/VGIIIC.
Cryptococcus neoformans s.s. has worldwide distribution and typically causes cryptococcal meningitis (CM) among hosts with impaired immunity. Previously, Cryptococcus gattii s.l. was believed to be geographically restricted to tropical and subtropical climates and associated with the immunocompetent hosts. In the last decade, reports of C. gattii s.l. infection from British Columbia, North West America, Brazil, Sub-Saharan Africa, Mediterranean Europe, India, and Australia have established the global distribution of the pathogen. Moreover, several studies have also documented that C. tetragattii (AFLP7/VGIV) and C. bacillisporus (AFLPS/VGIII) are pathogenic to both immunocompromised and immunocompetent hosts.

Isolated cases of C. gattii s.l. have been reported from southern and north-eastern part of India, but reports on large-scale molecular analysis of clinical isolates are lacking. From South India, we have earlier reported four cases of CNS infection due to C. tetragattii. The global occurrence of C. gattii s.l. was determined by analysing several studies on clinical, environmental and veterinary isolates and it was documented that AFLP4/VGI is the most frequent molecular type. Later, considering the potential sample biases, it has been concluded that C. deuterogattii is more common (47%) followed by C. gattii s.s. (34%), C. bacillisporus (11%) and C. tetragattii (8%).

Cryptococcus gattii s.l. is believed to cause mass lesions known as cryptococcoma among immunocompetent individuals, although clinical presentation may vary depending on the severity of the infection and immunological status of the patient.

In general, C. gattii s.l. has been found to be less susceptible than C. neoformans s.l. to commonly used antifungal agents except amphotericin B (AMB) and flucytosine. However, there is scarce data available on antifungal susceptibility of C. gattii s.l. from India.

With this background, we aimed to study the clinical features of CNS infection caused by C. gattii s.l., molecular epidemiology and antifungal susceptibility profile of the pathogen.

2 | MATERIALS AND METHODS

This prospective study was carried out in the department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, the largest tertiary neuro-care centre of India. Cases diagnosed to have CNS infection due to C. gattii s.l. from January 2012 to December 2015 were included in the study. The study was approved by the institute ethical committee. Clinico-demographic features were recorded in a semi-structured proforma (Table 1). The variables recorded were age, gender, immunological status, presenting illness, past medical history, comorbid factors and geographical location of the patient. Imaging features, CSF analysis, and microbiological results were also recorded.

2.1 | Diagnosis of cryptococcal infection

The diagnosis of cryptococcal infection was based on the presence of round capsulated budding yeast cells in India Ink staining, antigen detection by Cryptococcal antigen latex agglutination system (CALAS) kit (Meridian Bio Sciences, Cincinnati, Ohio, United States), and isolation of Cryptococcus from CSF on sabouraud dextrose agar (SDA) incubated at 25 and 37 °C. In one of the cases of cryptococcoma/abscess, Cryptococcus was identified by Gram staining and culture of the pus specimen. Creamy mucoid colonies on SDA medium were seen within 48–72 hours and identified as Cryptococcus by observing the characteristic micromorphological features. The isolates were confirmed as C. neoformans s.l./C. gattii s.l. based on phenol oxidase positivity on Niger seed agar and urease positivity. The cryptococcosis neoformans s.l. and C. gattii s.l. were primarily differentiated by chemotyping on canavanine-glycine-bromothymol blue media. All cryptococcal isolates were stocked in 20% glycerol for further analysis.

2.2 | CSF analysis

CSF cell count and analysis of biochemical parameters were performed for 19 cases (In one case pus sample was analysed). CSF glucose and protein levels were evaluated by automated methods using Hexokinase/Glucose-6-phosphate Dehydrogenase assay and Pyrogallol red assay respectively.

2.3 | Molecular characterization

The following C. gattii s.l. reference strains, representing each of the four molecular types, were used for the study. Cryptococcus gattii s.s. WM276 (serotype B), C. deuterogattii R265 (serotype B) and C. bacillisporus NIH312 (serotype C, VGIII) was kindly provided by Dr. Kaustav Sanyal, Molecular Mycology Division, Jawaharlal Nehru Center for Advance Scientific Research, Bangalore (India) and C. tetragattii WM779 (serotype C) was a kind donation by Prof. Joseph Heitman, Duke University, USA. These reference strains also served as internal controls for the reproducibility of typing technique.

2.4 | Genomic DNA extraction

The cryptococcal cultures were grown on SDA plate containing 1 mg/mL chloramphenicol for 48 hours at 35 °C. DNA was extracted using AmPurE fungal genomic DNA extraction kit (Juniper Life Sciences, Bangalore, India) method. Purified DNA was stored at ~20 °C.

2.5 | Differentiation between Cryptococcus gattii species complex and Cryptococcus neoformans species complex

PCR primer pair CNa-70-S and CNa-70-A as described by Casali et al. were used to amplify a specific DNA fragment from C. neoformans s.l. and the PCR primer pair CNa-49-S and CNa-49-A was used to amplify a DNA fragment from C. gattii s.l. Amplification reactions were performed in a volume of 25 μL in Applied Biosystem Veriti Thermal Cycler, as described by Casali et al. All amplified products were characterized by electrophoresis on 2% agarose gels in 1X TAE buffer 80 V for 90 minutes and then stained with a solution of ethidium bromide at 0.5 μg/mL.
<table>
<thead>
<tr>
<th>S no</th>
<th>Age in years/Gender</th>
<th>Place (State)</th>
<th>Comorbid factors</th>
<th>HIV/CD4</th>
<th>Diagnosis</th>
<th>Geno-types</th>
<th>FLC sensitivity (MIC in μg/mL)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30/Male</td>
<td>Tamil Nadu</td>
<td>Tuberculous meningitis</td>
<td>Positive</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>S (&lt;1)</td>
<td>Succumbed</td>
</tr>
<tr>
<td>2</td>
<td>72/Male</td>
<td>West Bengal</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcoma</td>
<td>VGI</td>
<td>S (1)</td>
<td>Succumbed after 1 mo</td>
</tr>
<tr>
<td>3</td>
<td>32/Male</td>
<td>Karnataka</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>S (2)</td>
<td>Succumbed</td>
</tr>
<tr>
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<td>30/Male</td>
<td>Bihar</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcoma</td>
<td>VGI</td>
<td>S (4)</td>
<td>Recovered</td>
</tr>
<tr>
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<td>17/Female</td>
<td>Karnataka</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>S (2)</td>
<td>Recovered</td>
</tr>
<tr>
<td>6</td>
<td>20/Female</td>
<td>Karnataka</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>R (32)</td>
<td>Recovered</td>
</tr>
<tr>
<td>7</td>
<td>15/Female</td>
<td>Tamil Nadu</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>R (16)</td>
<td>Recovered</td>
</tr>
<tr>
<td>8</td>
<td>26/Male</td>
<td>Karnataka</td>
<td>Tuberculous meningitis</td>
<td>Positive</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>I (8)</td>
<td>Succumbed</td>
</tr>
<tr>
<td>9</td>
<td>26/Female</td>
<td>Karnataka</td>
<td>No comorbidity</td>
<td>Positive</td>
<td>Cryptococcal meningitis</td>
<td>VGIII</td>
<td>R (32)</td>
<td>Succumbed after 3 d</td>
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<td>10</td>
<td>25/Male</td>
<td>Andhra Pradesh</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>I (8)</td>
<td>Succumbed after 20 d</td>
</tr>
<tr>
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<td>No comorbidity</td>
<td>Positive</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>I (8)</td>
<td>Succumbed after 15 d</td>
</tr>
<tr>
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<td>30/Female</td>
<td>Tamil Nadu</td>
<td>No comorbidity</td>
<td>Positive</td>
<td>Cryptococcoma</td>
<td>VGI</td>
<td>S (4)</td>
<td>Succumbed</td>
</tr>
<tr>
<td>13</td>
<td>26/Male</td>
<td>Karnataka</td>
<td>Tuberculous meningitis</td>
<td>Positive</td>
<td>Cryptococcoma</td>
<td>VGI</td>
<td>S (&lt;1)</td>
<td>Succumbed in 2 d</td>
</tr>
<tr>
<td>14</td>
<td>37/Male</td>
<td>Karnataka</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>S (2)</td>
<td>Succumbed after 2 mo</td>
</tr>
<tr>
<td>15</td>
<td>32/Male</td>
<td>Karnataka</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>R (16)</td>
<td>Succumbed</td>
</tr>
<tr>
<td>16</td>
<td>49/Male</td>
<td>Karnataka</td>
<td>Diabetes, Tuberculous meningitis</td>
<td>Positive</td>
<td>Cryptococcoma</td>
<td>VGI</td>
<td>S (&lt;1)</td>
<td>Succumbed</td>
</tr>
<tr>
<td>17</td>
<td>63/Male</td>
<td>Karnataka</td>
<td>No comorbidity</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>R (16)</td>
<td>Succumbed after 10 d</td>
</tr>
<tr>
<td>18</td>
<td>68/Male</td>
<td>Karnataka</td>
<td>Lymphocytic leukaemia</td>
<td>Negative</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>S (1)</td>
<td>Succumbed after 2 mo</td>
</tr>
<tr>
<td>19</td>
<td>48/Female</td>
<td>Karnataka</td>
<td>Tuberculous meningitis</td>
<td>Positive</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>I (8)</td>
<td>Succumbed</td>
</tr>
<tr>
<td>20</td>
<td>41/Male</td>
<td>Karnataka</td>
<td>Diabetes mellitus</td>
<td>Positive</td>
<td>Cryptococcal meningitis</td>
<td>VGI</td>
<td>I (8)</td>
<td>Succumbed</td>
</tr>
</tbody>
</table>

*All the isolates were sensitive to AMB, 5-FC, and VOR (not shown on the table).
2.6 Molecular genotyping by PCR fingerprinting

Primers of the microsatellite-specific sequences (GTG)$_3$ were used as single primers in the PCR, in a slightly modified method originally described by Meyer et al. The amplifications were performed in a final volume of 50 μL, containing 15 μL sterile deionized PCR grade water, 27.5 μL 1X PCR master mix (New England Biolabs, Inc, Massachusetts, USA) containing 20 mmol/L Tris-HCl, 22 mmol/L KCl, 22 mmol/L NH$_4$Cl, 1.8 mmol/L MgCl$_2$, 5% Glycerol, 0.2 mmol/L dNTPs, 1X Xylene Cyanol, 1X Tartrazine, 25 units/mL Taq DNA Polymerase (pH 8.9) and 50 ng primer.

PCR amplification was performed in 40 cycles using denaturation at 94 °C for 20 seconds, annealing at 50 °C for 1 minute, extension at 72 °C for 20 seconds and a final extension cycle at 72 °C for 6 minutes. Amplified products were separated by electrophoresis in 1.6% agarose gel containing ethidium bromide (0.3 mg/mL), in 1X Tris-Acetate EDTA (TAE) buffer at 55 V for 4 hours. The bands were visualized under UV light in Syngene Gel Documentation system. The molecular types (VGI–VGIV) were assigned by comparing with the reference strains loaded on each gel.

2.7 Determination of mating type

Two PCR primer pairs specific for mating type α and α of C. neoforms/gattii, were used to detect the mating type, as described by Chaturvedi et al. The PCR products were separated on 2% agarose gels in 1X TAE buffer, stained with ethidium bromide at 80 V.

2.8 Antifungal susceptibility test

Susceptibility of the isolates to AMB, fluconazole (FLC), voriconazole (VOR), and 5-flucytosine (5-FC) was performed by VITEK 2C automated method. Candida krusei ATCC6258 and Candida parapsilosis ATCC22019 were used for quality control in this experiment. Minimum inhibitory concentration (MIC$_{90}$) were determined by the VITEK2 system.

2.9 Analysis of the clinical presentation

The clinical manifestations of both cryptococcoma and CM cases were analysed and shown in Table 2. Statistical analysis was performed by Fischer’s Exact test in SPSS software version 22.0 (IBM Analytics, North Castle, New York, United States) to determine the P values.

3 RESULTS

A total of 199 cases of CNS cryptococcosis were diagnosed from 2012 to 2015; out of which 179 (89.9%) were Cryptococcus neoforms s.l. and 20 (10.1%) were C. gattii s.l. Out of these 20 cases 15 (75%) were CM and five (25%) were cryptococcoma; 10 (50%) immunosuppressed (nine HIV sero positive and one case of leukaemia).

3.1 Demography

Age group of the patients ranged from 15 to 72 years. In both cryptococcoma and meningitis, there was male preponderance; 13 (65%) were male with mean age of 40.8 years (σ=16.7) and seven (35%) were females with a mean age of 26.4 years (σ=11.1) (Table 1).

3.2 Clinical findings

Headache (100%), neck rigidity (73.3%-80%), vomiting (60%-80%), impairment of vision (40%-60%) were the predominant clinical

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No of cases (%)</th>
<th>Cryptococcoma (%)</th>
<th>Meningitis (%)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>13 (65%)</td>
<td>4 (80%)</td>
<td>9 (60%)</td>
<td>.61*</td>
</tr>
<tr>
<td>Female</td>
<td>7 (35%)</td>
<td>1 (20%)</td>
<td>6 (40%)</td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>20 (100%)</td>
<td>5 (100%)</td>
<td>15 (100%)</td>
<td>1.00*</td>
</tr>
<tr>
<td>Fever</td>
<td>14 (70%)</td>
<td>0 (0%)</td>
<td>14 (93.3%)</td>
<td>.004*</td>
</tr>
<tr>
<td>Vomiting</td>
<td>13 (65%)</td>
<td>4 (80%)</td>
<td>9 (60%)</td>
<td>.61*</td>
</tr>
<tr>
<td>Neck rigidity</td>
<td>15 (75%)</td>
<td>4 (80%)</td>
<td>11(73.3%)</td>
<td>1.0*</td>
</tr>
<tr>
<td>Visual disturbances</td>
<td>9 (45%)</td>
<td>3 (60%)</td>
<td>6 (40%)</td>
<td>.61*</td>
</tr>
<tr>
<td>altered behaviour</td>
<td>10 (50%)</td>
<td>0 (0%)</td>
<td>10 (66.7%)</td>
<td>.032*</td>
</tr>
<tr>
<td>C. tetragattii VGIV</td>
<td>15 (75%)</td>
<td>3 (60%)</td>
<td>12 (80%)</td>
<td>.27*</td>
</tr>
<tr>
<td>C. gattii s.s VGI</td>
<td>4 (20%)</td>
<td>2 (40%)</td>
<td>2 (13.3%)</td>
<td></td>
</tr>
<tr>
<td>C. bacillisporus VGIII</td>
<td>1 (5%)</td>
<td>0</td>
<td>1 (6.7%)</td>
<td></td>
</tr>
</tbody>
</table>

P values determined by Fisher’s exact test. $^{*} P < .05$ is considered as statistically significant difference between cases of cryptococcoma and meningitis; $^{*} P > .05$ is considered statistically insignificant.
manifestation common to both meningitis and cryptococcoma cases. Fever (93.3%) and behavioural abnormalities (66.7%) were exclusively observed in meningitis cases (Table 2).

3.3 Imaging features

Out of the 20 patients who were included in this study, 11 had computed tomographic (CT) scan of the brain for evaluation, two had Magnetic Resonance (MR) images and seven had both CT and MR images (Figure 1).

Diffuse leptomeningeal enhancement was the commonest finding in seven (35%) patients. This leptomeningeal enhancement was predominantly sulcal in distribution. The basal meninges were relatively spared. Out of these seven patients, one had communicating hydrocephalus secondary to meningitis induced impairment of CSF flow. Two had non-enhancing hypodensities in the right caudate nucleus head.

The next most common finding was multiple small T1 hypointense, T2 and fluid attenuation inversion recovery hyperintense lesions along the perivascular spaces predominantly in bilateral basal ganglia and few in supratentorial white matter with no diffusion restriction or contrast enhancement suggestive of cryptococcoma. These were seen in four (20%) of subjects, out of which two had co-existence of diffused meningitis. Another patient (5%) also had a mass like lesion in the right cerebellar hemisphere with irregular peripheral ring enhancement and intracavitary nodular projections. No diffusion restriction was seen within the lesion to suggest abscess formation.

One patient (5%) had multiple small abscesses in bilateral parietal regions with perilesional oedema. These lesions were T1 hypointense and had alternating rings of hypo and hyperintensity in T2 with diffusion restriction and peripheral ring enhancement.

Two patients had only calcified granulomas. Imaging features in the remaining five subjects were normal.

3.4 CSF analysis

CSF was analysed in 19 cases (15 cases of meningitis and four cases of cryptococcoma). The CSF cell count ranged from nil to 320 cells/mm³ with an average of 70 cells/mm³ (σ=95) among 18 cases of CM, nine to 34 cells/mm³ with an average of 21.5 cells/mm³.

**FIGURE 1** Figure shows imaging of cryptococcal meningitis and cryptococcoma cases: (A) Axial CT image of a proven case of cryptococcosis with diffuse meningeal enhancement. (B, C) Axial T2W images at the level of basal ganglia shows multiple small T2 hyperintense foci predominantly in the caudate head and lentiform nucleus bilaterally without contrast enhancement (not shown) suggestive of cryptococcoma. (D) Cryptococcoma formed mass lesion in the cerebellar hemisphere
(σ=10.4) among cryptococcoma and in one case of lymphocytic leukaemia the cell count was 1100 cells/mm². The predominant cell type was lymphocytes in both meningitis and cryptococcoma cases. CSF analysis was not performed for one case of cryptococcoma, who underwent surgical removal of the lesions and pus sample was collected.

CSF glucose was lower than normal in 10 (66.7%) and two (50%) cases of meningitis and cryptococcoma respectively. CSF protein level was higher than normal range (15-45 mg/dL) in 18 (94.7%) cases and normal in one case of meningitis (Figure 2).

3.5 | Genotype and mating type analysis

Among the total 199 isolates, 20 were confirmed as *C. gattii* s.l. by the variety determination PCR. Out of these 20 isolates, 15 (75%) were detected as *C. tetragattii* and four (20%) were *C. gattii* s.s. followed by one (5%) *C. bacillisporus* by PCR fingerprinting. *Cryptococcus tetragattii* was predominant molecular type among both the cases of CM (80%) and cryptococcoma (60%). *C. gattii* s.s. was identified from 13.3% CM and 40% of cryptococcoma cases.

All the isolates were mating type α.

3.6 | Antifungal susceptibility

All the 20 isolates (100%) were sensitive to AMB (MIC₉₀: 0.25≤1 μg/mL), 5-FC (MIC₉₀: 1 μg/mL), and VOR (MIC₉₀: 0.12≤0.5 μg/mL). Fluconazole sensitivity had a varied pattern with 10 (50%) of the isolates being sensitive (MIC₉₀: 1≤4 μg/mL), five (25%) resistant (MIC₉₀: 16≤32 μg/mL) and the rest five (25%) with intermediate sensitivity (MIC₉₀: 8 μg/mL). Sensitivity was interpreted by the VITEK2 system and the MIC values were compared with epidemiological cut-off values (ECV) reported by Espinel-Ingroff et al.¹⁹ for all four antifungal agents (Table 3).

Notably, all the isolates from cases of cryptococcoma were sensitive to all antifungal agents and the isolates that were resistant and intermediate sensitivity to FLC were from CM.

3.7 | Treatment

The patients were started on intravenous therapy of AMB 0.5 mg/kg body weight/day and gradually increased up to 1 mg/kg body weight/day in combination with oral FLC 400 mg/day (or 5-FC 100 mg/kg body weight/day) for 2 weeks followed by a maintenance therapy of FLC 400 mg/day for 3-6 months or more.²⁰ Maintenance therapy with 5-FC 100 mg/kg body weight/day was advised to six cases. Two patients with cryptococcoma had underwent surgical removal of the lesion followed by standard antifungal treatment.

3.8 | Outcome

In both cryptococcoma and meningitis cases mortality was 80%. Among the four cases who survived, one was cryptococcoma who underwent surgery and rest of three cases of meningitis were seen in the out-patient department on a regular basis for a period of 1 year and later was lost for follow up.

Among the successfully treated cases, one was caused by *C. gattii* s.s. and three were due to *C. tetragattii*.

3.9 | HIV status and *C. gattii* s.l. infection

Seven (46.7%) among 15 cases of CM, and three (60%) out of five cases of cryptococcoma were immunocompromised. Among HIV positive cases, age group ranged from 26-49 years irrespective of CM or cryptococcoma. Among HIV negative cases age group ranged from 15-72 years, which include two cases of CM in children aged 15 years and 17 years.

![Figure 2](image-url)  
**Figure 2** Figure showing protein and glucose levels in CSF.
Vomiting and altered behaviour were prominent in immunocompetent whereas visual difficulties were more common in immunocompromised hosts.

Among immunosuppressed cases eight (80%) were C. tetragattii and one (10%) each of C. gattii s.s. and C. bacillisporus. Among the immunocompetent cases seven (70%) were C. tetragattii and three (30%) were C. gattii s.s.

The five FLC resistance cases, one was immunocompromised and rest four were immunocompetent.

Immunosuppressed patients had 100% mortality within 2 days to 6 months’ post-diagnosis. Four (40%) immunocompetent cases survived after primary and follow-up therapy.

4 | DISCUSSION

Cryptococcus gattii s.l. was not considered as an important pathogen till recently because the infection by this organism represented only 1% of the cryptococcosis cases worldwide.21 Even in endemic area like Australia, the reported infection rate was only 0.94 cases per million.22 The outbreak on Vancouver Island of British Columbia (Canada) in 1999, has expanded its epidemiology in temperate regions.23 Till then C. gattii s.l. has been reported from Columbia23, Papua, New Guinea,24 Venezuela,25,26 French Guiana,27 Vietnam,29 Hong Kong,29 Botswana,20 Zimbabwe16 and Malawi.23 On the other hand, the overall prevalence was found to be lower in Europe, South Africa, Mexico, Argentina, and Asia including China (without Hong Kong), southeast Asia and India.12,23

There are few reports of clinical isolates of C. gattii s.l., which documents prevalence rate ranging from 2.8% in south India to 8%-8.8% in north India.7,22-24 The present series records the increased prevalence of 10.1% cases, as compared to our previous reports, which is probably a consequence of alterations in the ecology and biology of this pathogen.

Most of our patients (15; 75%) presented with meningitis. Morgan et al.25 also report C. gattii s.l. meningitis in their study. Among our cases young adults (26-45 years) were commonly affected by both cryptococcoma and meningitis. There were two (10%) cases below the age of 18 years who were immunocompetent. A high proportion of CNS cryptococcosis due to C. gattii s.l. among immunocompetent young individuals have also been documented in Colombia.23 There was male preponderance probably due to increased exposure of males to environmental sources.36

Cryptococcus gattii s.l. causes potentially fatal infection both as opportunistic and primary pathogen.2 Several factors such as oral corticosteroids, chemotherapy, older age may increase the risk of infection by this species.37 In our study 10 (50%) cases were immunocompromised which was similar to the report by Morgan et al.,35 who have documented 61% cases of C. gattii s.l. infection to be HIV seropositive, supporting the fact that C. gattii s.l. is no longer an exclusive etiologic agent for immunocompetent individuals.5 Headache, fever, and neck stiffness were the most salient features and were common to both cryptococcoma and meningitis patients. Fever (P=.004) and altered behaviour (P=.032) were prominent among meningitis cases.

The most common imaging finding in these patients was diffuse leptomeningeal enhancement (35%) followed by cryptococcoma and lesion in 25% of the subjects. All the patients, who had visual difficulties, the radiological findings featured either presence of abscess, ring enhancing/hypodense lesions or meningeal infiltration.

Most of the clinical isolates from Europe, Asia and Australia are C. gattii s.s. whereas in North and South America C. deuterogattii and C. bacillisporus are more prevalent.3 Cryptococcus tetr gattii is predominant in southern part of Africa5,31 and also reported from India, Mexico, Colombia, Puerto Rico, Spain.5,25,38-40 Although in India, C. gattii s.l. has been isolated from the environment,33 there are limited reports of clinical isolation, of which only two mention the molecular types of the isolates. Jain et al.26 have reported five isolates of C. deuterogattii, and Chowdhary et al.33 have mentioned two clinical isolates of C. gattii s.s. The present finding agrees with our previous report7 as majority of our C. gattii s.l. isolates are C. tetr gattii and the patients hailed from three states: Karnataka, Tamilnadu and Andhra Pradesh, from southern part of India indicating the probable predominance of C. tetr gattii in this part of the country. C. gattii s.s. isolated from four (20%) cases came from different states; Karnataka, Tamil Nadu (south India), West Bengal and Bihar (east India). Thus, it appears that C. gattii s.s. is widely distributed in India.5,33 We had one case of CM due to C. bacillisporus from Karnataka, which is probably the first report from India, to the best of our knowledge.

During the Vancouver Island outbreak, due to C. deuterogattii, the most common clinical presentation was meningitis.23 In this study, C. tetr gattii was more common among both meningitis and cryptococcoma patients although there was no statistically significant difference between the molecular types involved and disease presentation (P=.27) (Table 2). Cryptococcus tetr gattii was also predominant species among both immunosuppressed (80%) and immunocompetent (70%) individuals and C. gattii s.s. was more common among

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<th><strong>TABLE 3</strong> In vitro antifungal susceptibility test of C. gattii (n=20); MIC values expressed in µg/mL</th>
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<tr>
<td><strong>Antifungals tested</strong></td>
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<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>Amphotericin B</td>
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<tr>
<td>Fluconazole</td>
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<tr>
<td>5-Flucytosine</td>
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<td>Voriconazole</td>
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<sup>a</sup>ECV for the C. gattii s.l isolates were acquired from reports published by Espinel-Ingroff et al.19

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LAHIRI MUKHOPADHYAY et al.
immunocompetent patients (75%). Most of the clinical and environmental isolates worldwide belong to mating type α,7,41 as were all our isolates.

The isolates were tested for the susceptibility to four most commonly used antifungal agents. MIC90 and the sensitivity pattern was generated by VITEK2 system42 which was comparable to ECVs for all the antifungals reported by Espinel-Ingroff et al.19,43 Thompson et al.44 described in their study that C. gattii s.l. exhibits low susceptibility to FLC, which supports our present study where five (25%) of the isolates were resistant and five (33.3%) showed intermediate susceptibility to FLC.

Four (26.7%) of the 15 C. tetragattii and the C. bacillisporus isolates were resistant to FLC. An investigation from USA describing FLC resistance of C. gattii, also showed C. tetragattii (Colombia) and C. bacillisporus (Australia, USA) isolates from several sources were resistance to FLC,45 which explains that FLC resistance is present in C. bacillisporus and C. tetragattii in endemic areas. All the four (100%) C. gattii s.s. isolates were sensitive to all the antifungal drugs tested including FLC, which supports the previous study by Chowdhary et al.33 Among the four patients who survived the infection, two were due to C. tetragattii FLC resistant isolates, who had undergone long-term maintenance therapy with 5-FC, rest of the immunocompetent cases could not survive because they visited the hospital in the advanced stage of the disease. A recent review suggests that treatment of C. gattii s.l. infection comprises of induction therapy with AMB and 5-FC for 2-6 weeks followed by maintenance therapy with FLC for 6-12 months can eradicate the infection.3 Thus, FLC resistance remains a factor of concern for both the clinicians and patients.

5 | CONCLUSION

Cryptococcus gattii s.l. can cause meningitis or cryptococcoma in both immunocompromised and immunocompetent individuals. Cryptococcus tetragattii is probably more prevalent in south India and C. gattii s.s. is well distributed in the country. We report the first clinical case of meningitis caused by C. bacillisporus. Fluconazole resistance was documented among C. tetragattii and C. bacillisporus isolates and C. gattii s.s. isolates were sensitive to all antifungal agents. It is important to understand the emergence and the geographic distribution of this important fungi as the geographic variation in the distribution of this fungus is striking.

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CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

ETHICAL STATEMENT

This study was approved by institutional ethics committee of National Institute of Mental Health and Neuro Sciences, Bangalore, India.

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