

Should cryptococcal antigen screening be considered as a routine procedure in antiretroviral therapy naïve severely immunocompromised HIV-seropositives – a prevalence study from Eastern India to support recent 2018 WHO guidelines

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Abstract

Introduction: Cryptococcal meningitis, a leading opportunistic infection, causes significant morbidity and mortality in people with advanced human immunodeficiency virus (HIV). It accounts for an estimated 15% of acquired immune deficiency syndrome-related deaths globally. As recommended by the World Health Organization (WHO) 2018 guidelines, this invasive disease is preventable by routine cryptococcal antigen (CrAg) screening of all advanced HIV patients followed by pre-emptive antifungal therapy. An estimate of disease prevalence in antiretroviral treatment (ART)-naïve HIV-positive adult Indian population is essential to include this in routine screening strategy. We estimated CrAg prevalence as a guiding resource in a public health approach.

Material and methods: The study design was longitudinal. ART naïve HIV-seropositive patients with CD4 count ≤ 100 cells/ μl , attending ART center at the School of Tropical Medicine, Kolkata, India were screened for CrAg using both latex agglutination and lateral flow assay kits. A total of 390 subjects were enrolled into the study, and evaluated for association of CrAg with age, sex, CD4, presence of opportunistic infections, WHO HIV staging, and clinical symptoms.

Results: Of 390 subjects tested, the median CD4 count was 42 cells/ μl in CrAg-positive and 46 in CrAg-negative patients. Median (IQR) age of all participants was 40 (range, 34-46) years. CrAg positivity was 12.56%, comparatively higher in those with CD4 ≤ 50 cells/ μl . Asymptomatic patients had CrAg positivity of 4.6%. Statistically significant association was noted with male sex ($p = 0.03$), triad symptoms of fever, headache, vomiting ($p = 0.013$), and altered mental status ($p = 0.033$).

Conclusions: This study aims to estimate CrAg prevalence in India to justify the need for routine screening and pre-emptive treatment in advanced HIV infection. Incorporating this screening would definitely reduce the risk of cryptococcus meningitis-induced mortality and morbidity, as recommended by the WHO guidelines.

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Introduction

Cryptococcal meningitis (CM) has been recognized as a leading cause of adult human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS)-related mortality in sub-Saharan Africa. Data relating to the prevalence of this potentially preventable infection is sparse in South Asian countries, specifically in India. Due to paucity of prevalence data, its prevention and treatment remains ill-defined and does not get prioritized. Mortality from CM is highest in developing low-income countries, accounting for around 70-80% in low-income versus only 20-30% in high-income countries [1].

CM is known as a “sleeping disease” and is being predicted as a “mycosis of future”, but due to resource constraints and the uncertainty related to the prevalence of CM data, routine screening, evaluation, and pre-emptive treatment of pre-antiretroviral therapy (ART) subjects for cryptococcal infection are not a part of the National Program in India [2, 3].

As the mortality of CM is quite high, special attention is needed for subjects who present to antiretroviral clinics with advanced HIV infection and exceptionally low CD4 count. Data from retrospective hospital-based studies conducted in Brazil and Argentina show a high case fatality, ranging from 26% to 63% [4].

The high case fatality is related to a delay in presentation with late diagnosis. Non-availability of rapid diagnostic kits, poor accessibility, or contraindications to invasive procedures such as lumbar puncture, are the most common reasons for the delay in diagnosis [5].

CM is one of the leading opportunistic fungal infections and AIDS-defining illness in patients with CD4 count ≤ 100 cells/ μl . It is caused by the encapsulated yeast of *Cryptococcus neoformans* or *C. gattii* [6]. Among these two, *C. neoformans* is more commonly associated with meningitis in immunocompromised patients. Cryptococcal antigenemia is considered an independent predictor of CM in HIV subjects with advanced immunosuppression [7].

In a meta-analysis, Lawn *et al.* observed that in sub-Saharan Africa, CM accounts for almost 20% of mortality in subjects with AIDS [8]. In a cross-sectional study from Namibia, Sawadogo *et al.* reported an estimated prevalence of 3.3% cryptococcal antigen (CrAg) positivity on de-identified plasma specimens with CD4 ≤ 100 cells/ μl [9]. According to meta-analysis by Lawn, almost 8 to 26% of deaths in people living with HIV (PLHIV) occurred in the first few months after detection and initiation of ART, of which 20% accounted for CM [8]. In Southeast Asia, CrAg prevalence in patients with CD4 ≤ 100 cells/ μl is reported between 4.0% and 20.6% in both clinically suspected and asymptomatic PLHIV. In few other studies, the prevalence has been observed as high as 12.9%, where only asymptomatic, ART-naïve patients were enrolled [10-12]. The prevalence pattern is slightly more variable in India. In a study from New Delhi, North India, the prevalence of cryptococcal infection detected by conventional method (India ink and culture) was documented as high as 46% in a cohort of HIV-positive

meningitis patients [13]. In a recent study by Anuradha *et al.* from New Delhi, latex agglutination (LA) tests of serum samples in ART naïve subjects with CD4 ≤ 100 cells/ μl yielded a prevalence of 3.125% [14].

The World Health Organization (WHO) in its updated version of March 2018, strongly recommends routine CrAg screening of HIV-infected adults and adolescents with CD4 count ≤ 100 cells/ μl before ART initiation, and has also considered screening at a higher CD4 cell count threshold of < 200 cells/ μl . If cryptococcal antigen screening is not available, fluconazole primary prophylaxis has been advised for all individuals with CD4 count ≤ 100 cells/ μl , with special consideration at a higher CD4 count of < 200 cells/ μl . If CrAg-positive detected, as a strategy to reduce the development of CM, pre-emptive antifungal therapy as induction, consolidation, and maintenance treatments are recommended [15]. Previously, this screening strategy was recommended by the WHO only if the prevalence of CrAg in an area was $> 3\%$.

The primary objective of this study was to ascertain the prevalence of CrAg positivity among ART-naïve, HIV-seropositive patients, with CD4 count of ≤ 100 cells/ μl .

Material and methods

This longitudinal study was conducted at the Centre of Excellence in HIV Care, School of Tropical Medicine, Kolkata, India, between January 2014 to July 2017. Whole blood samples were drawn from 390 ART-naïve, HIV-seropositive subjects, who had CD4 count ≤ 100 cells/ μl .

An approach of “reflex testing” was adopted for this study by which, serum CrAg testing was done on a day-to-day basis by selecting the ART-naïve subjects with CD4 counts of ≤ 100 cells/ μl . Inclusion criteria included ART-naïve, HIV-seropositive adults and adolescents above 15 years of age, and written, informed consent was obtained from every participant before the enrollment. To eliminate selection bias, history and clinical examination were completed and documented only after the recruitment. A thorough history and detailed clinical examination was done for all recruited subjects, and medical records were accessed for data collection.

Baseline absolute CD4 count, WHO clinical staging for HIV/AIDS, and demographic variables were recorded. Patients who had past or ongoing history of cryptococcal disease or meningitis were excluded.

The subjects were screened for CrAg by LA using CALAS kits (Meridian Bioscience Inc., OH, USA) and lateral flow assay (LFA), namely IMMY dipsticks (Immuno Mycologics, Norman, OK, USA). LFA is an immuno-chromatographic sandwich dipstick assay. Both kits are capable of detecting the capsular polysaccharide of *Cryptococcus neoformans* in serum. A comparative parallel testing was done using both kits for each sample.

Modern cryptococcal antigen tests are extremely sensitive and specific [16, 17]. CrAg is detectable around 21 days prior to symptoms of CM and it has been observed that in 11% of cases, CrAg is tested positive at 100 days prior to actu-

al clinical symptoms [18]. This window period is important for primary treatment to reduce mortality associated with this meningitis [19]. In our study, the results of LFA were interpreted according to manufacturer instructions and recorded on a standard laboratory test result form with a scale ranging from negative to 4+ positive. LFA is an overly sensitive, specific, and a point-of-care rapid diagnostic test, which needs no infrastructure or training [20, 21]. Testing for LFA was performed with IMMY LFA kits according to the manufacturer instruction. One line was interpreted as negative and two lines were considered as positive. To enumerate the predictors of CrAg positivity, a nested case-control model was used. In this, the controls were randomly selected from CrAg-negative subjects in a 4 : 1 ratio against CrAg-positive cases. At this point, the CrAg-positive patients were labelled as “cases” and CrAg-negative as “controls”.

Statistical analysis

The study results were collected manually, and all variables entered into a standardized database. CrAg prevalence was stratified by sex, age, race, education, WHO clinical staging, coexistence of other infections, and demographic parameters. Mean, median, and standard deviation were calculated for both the cases and controls. The association between CrAg positivity and these variables were analyzed using SPSS software version 20.

Results

A total of 390 eligible subjects were screened. Of them, 49 patients (12.56%) were tested positive for CrAg by both the test kits. No discordance was encountered with the two different test kit (LA and LFA) results. In other words, all those who tested CrAg-positive with LFA were also positive with LA kit.

Within a range of 1-100, the median CD4 count was 42 cells/ μ l in CrAg-positive cases and 46 cells/ μ l in CrAg-negative controls. CrAg positivity was comparatively higher in the subgroup of CrAg-positive cases, with CD4 < 50 cells/ μ l (59.1%) as compared to the range of 50-100 cells/ μ l (40.8%).

Of all subjects tested, 81.3% were males, and approximately 92% of CrAg-positive patients were male. The median age for the positive subjects was 38 years (Table 1). Heterosexual transmission was the commonest mode for HIV infection. The majority (42.8%) of CrAg-positive cases were from rural area and married (71.4%), with an education up to secondary level (69.4%). Initial clinical examination and staging revealed that more than 80% of those who were later detected as CrAg-positive were of WHO HIV clinical stage 3 or 4.

The presence of only fever could not be correlated with CM, but the triad symptoms of fever, headache, and vomiting had a strong association with CrAg positivity.

Tuberculosis and oro-esophageal candidiasis were the commonest opportunistic infections. In CrAg positive patients, oral candidiasis was noted in 21 (42.8%) and esophageal candidiasis was documented in 5 cases (10.2%). Active TB

was more prevalent among CrAg-negative patients (44.4%), as compared to CrAg-positive subjects (32.6%) (Table 1).

Following the WHO December 2011 guidelines, all 38 consented CrAg-positive patients were treated with an induction therapy of intravenous amphotericin B for 2 weeks with high-dose of oral fluconazole, followed by oral fluconazole in consolidation phase for 8 weeks (if CM symptoms were present) or pre-emptively with only oral fluconazole (if CM symptoms were not evident). Not all of 11 CrAg-positive cases who had declined the empirical therapy were asymptomatic. Six presented a history of low-grade fever for more than 3 weeks, associated with headache, cough, and vomiting. Of these 11 positive patients declining therapy, 6 were diagnosed with TB meningitis and were started on anti-TB drugs as per national guidelines (Table 2). Among the rest five patients with CrAg positivity, three had a history of headache with convulsions and underwent an MRI with gadolinium contrast; they were diagnosed as toxoplasmosis of brain and positive anti-toxoplasma IgG. Among those who declined antifungal therapy, only 2 patients were truly asymptomatic, and they were reluctant to get admitted for the empirical antifungal treatment, as they thought they were apparently doing well. Though most, that is 75% of 49 positive cases were either 3+ or 2+ in LA test titre (Table 3) but according to the existing NACO guidelines for treatment of opportunistic infections, treatment is not offered to any asymptomatic cryptococcal-positive subjects.

Immediate ART initiation is not recommended for CrAg-positive HIV-seropositive patients as, according to WHO guidelines, there is a high-risk of mortality due to development of central nervous system (CNS) immune reconstruction inflammatory syndrome (IRIS). The median time to ART initiation from date of CrAg testing was 28 days (range, 14-42 days). ART was postponed by 2 weeks in 3 subjects who were CrAg-positive, relatively asymptomatic, and received oral fluconazole only. ART initiation was delayed by 3-6 weeks in CM-confirmed patients who received amphotericin B and high-dose of fluconazole. The compliance to amphotericin B and high-dose of fluconazole was 100%, and ART adherence was mostly above 95%. Following the induction and consolidation phase, the maintenance treatment with only oral fluconazole was advised until the CD4 count increased to level of above 200 cells/ μ l. Cerebrospinal fluid (CSF) evaluation was done in 22 of 49 CrAg-positive (44.8%) cases (Table 4). In the rest of patients, it was either not completed due to operational issues or to subjects' refusal to perform the test.

All enrolled subjects were followed up for 12 months and the outcomes of CM mortality or relapse were recorded. Among the patients who were treated for cryptococcal infection, 8 (21%) subjects died but among the survivors, none had a relapse within 12 months of follow-up (Table 2).

Among the 11 odd subjects who declined empirical treatment, one week following ART initiation, two of them were hospital admitted with severe meningitis at 14 weeks and 21 weeks from date of CrAg testing.

We observed a 12.56% of prevalence of CrAg positivity among the ART-naïve, HIV-positive subjects with CD4

Table 1. Baseline demographic and clinical characteristics of HIV-infected adults screened for cryptococcal antigen

Results	CrAg-positive, n = 49 (12.56%)	CrAg-negative, n = 341 (87.43%)	Total tested, n = 390	p-value (nested case-control model)
Male	45 (91.8%)	272 (79.8%)	317 (81.3%)	0.033
Female	4 (8.2%)	69 (20.2%)	73 (18.7%)	
Age (in years)				
Mean	40.84	40.72	40.74	0.440
Median	38.00	40.00	40.00	
Range	30-70	18-78	18-78	
IQR range	34-46	34-46	34-46	
CD4 range (cells/ μ l)				
< 50	29 (59.2%)	176 (51.6%)	205 (52.6%)	0.828
50-100	20 (40.8%)	165 (48.4%)	185 (47.4%)	0.781
CD4 (cells/ μ l)				
Mean	44.47	48.23	47.78	
Median	42.00	46.00	46.00	
Range	1-100	1-100	1-100	
IQR range	18-66	26-69	25-69	
Symptoms				
Fever (more than 4 weeks)	26/49 (53%)	220/341 (64.5%)	246 (n = 390)	0.142
Fever + headache + vomiting	26 (53%)	78 (22.9%)	104	0.013
Altered mental status (categorized as delirious/ somnolent/lethargic/stuporous/comatose)	5 (10.2%)	5 (1.46%)	10	0.033
Associated OI				
Tuberculosis	16 (32.6%)	151 (44.4%)	167	0.150
Candidiasis (oral and esophageal)	26 (53.0%)	194 (56.9%)	220	0.302
WHO clinical staging for HIV/AIDS				
1	3 (6.1%)	38 (11.1%)	41	0.406
2	5 (10.2%)	29 (9.2%)	34	
3	18 (36.7%)	118 (34.0%)	136	
4	23 (47.0%)	156 (45.7%)	179	

CrAg – cryptococcal antigen, IQR – interquartile range, OI – opportunistic infections

Table 2. Mortality data among cryptococcal antigen-positive cases (followed up for 12 months)

Number of CrAg-positive	49
Alive, n (%)	33 (67.3)
Death, n (%)	16 (32.7)
Median time to death	30 days
Range of days, within which death occurred	28-330 days (4 weeks to 47.1 weeks)
Highest number of deaths occurred	30 \pm 2 days
Treated with amphotericin B, fluconazole, or both	38; death among treated: 8 (21.05)
Not treated for cryptococcosis (both symptomatic and asymptomatic cases) who declined treatment, n (%)	11; death among non-treated: 8 (72.7)

\leq 100 cells/ μ l. The ART center had an average of 680 annual new ART initiators during the study period, of whom approximately 15% had CD4 count \leq 100 cells/ μ l. In these 3 years

of study period, average new ART initiations were recorded as 180,000 all over the country. If the prevalence data is extrapolated to estimate CrAg-positive subjects in ART-naïve PLHIV

all over India, which would roughly be 3,391 subjects who need pre-emptive treatment to reduce emergence of meningitis, with its morbidity and mortality.

Discussion

For a long time, diagnosis of CM was cumbersome and dependent on direct visualization under the microscope by India ink (nigrosine) staining or culture confirmation on Saboraud dextrose agar media. Though, the culture technique is the gold standard, but the long time needed for its reporting made early diagnosis and treatment difficult. With the advent of new antigen tests like enzyme immunoassay (EIA), LA, and the most recent LFA, it is much easier to obtain a rapid diagnosis. In particular, LFA is an easy, point-of-care, and relatively cheap test, which is a very useful tool for diagnosis in a resource-limited setting.

As a part of national guidelines for integral screening for HIV care, CrAg screening and treatment is presently implemented in several countries, including South Africa, Rwanda, Zimbabwe, Uganda, Namibia, and Mozambique. In a recently published meta-analysis, Nathan et al. have supported current recommendations to screen all PLHIV who have a CD4 count ≤ 100 cells/ μl for CrAg, and suggested that screening may be considered at CD4 cell count ≤ 200 cells/ μl [22]. WHO of 2018 guidelines have recommended cryptococcal screening followed by pre-emptive antifungal treatment for all HIV-positive adults and adolescents who present CD4 count of less than 100 cells/ μl as well as screening those with CD4 less than 200 cells/ μl . In places where this testing is not available, fluconazole primary prophylaxis has been advised.

In India, as per NACO guidelines, pre-emptive antifungal treatment is not recommended. Also, the program does not support a routine primary prophylaxis for subjects with CD4 of less than 100 cells/ μl . Hence presently, we are not able to estimate the benefits from preventive strategies, which is a debatable issue in a resource-limited country like India. This study showed a CrAg prevalence of 12.56% in de-identified serum samples. The prevalence of CrAg serum positivity in apparently symptomless subjects was 4.6% (18/390), which is closer to the global pooled prevalence of 6.4% observed in systematic review of 60 observational studies in subjects with CD4 < 100 cells/ μl [22].

The main objective of this 'reflex testing' method study was to yield valuable information whether the implementation of the screen-and-treat program would be effective to reduce mortality and morbidity in our country as a part of the National AIDS Control Program. For this, an assessment of the current prevalence in India would certainly help to evaluate the need for any of such recommendations. Adaptation of the point-of-care and the screen-and-treat strategies would need several key infrastructural services but would definitely be helpful in reducing morbidity and mortality.

In comparison to CrAg screening, the cost of treatment of symptomatic CM is substantially greater, but the relative morbidity and mortality is also high. On an average, the cost

Table 3. Latex agglutination (LA) serum titre result among cryptococcal antigen-positive cases, $N = 49$

Test result with LA	<i>n</i>
4+	10
3+	16
2+	21
1+	2

Table 4. Cerebrospinal fluid (CSF) analysis in 22 of 49 cryptococcal antigen-positive patients

Parameter	Characteristics ($n = 22$)	
Appearance	Clear	
Cell count (mm^3)	Median value: 24	Interquartile range: 14-250
Cell type	All lymphocytes	
Sugar (mg/dl)	Median value: 57	Interquartile range: 34-70
Protein(mg/dl)	Median value: 55	Interquartile range: 46-75
CSF to serum glucose ratio	> 0.6	
CSF CrAg test (LA kit method)	All positive ($n = 22$)	
CSF CrAg titer	Ranging from 1+ to 4+	

of LFA testing is Rs 350 (INR) or \$5 per person, and the cost of LA is Rs 300 (INR) or roughly \$4.2 per person. Pre-emptive treatment of CrAg-positive patients with amphotericin B and fluconazole poses less financial burden, as these drugs are available free of cost in the majority of government hospitals in India with antiretroviral centers. Therefore, the test-and-treat costs are relatively meagre as compared to no routine screening, with the in-patient treatment costs, morbidity, and mortality associated with meningitis. Patients often present with too advanced diseases to be effectively treated, leading to high mortality. The efficacy of this screening also needs to be evaluated as well as the cost of this test and treatment, which is the target of our follow-up studies.

According to the findings in the present study, with a high prevalence of CrAg positivity of around 12.6% in ART-naïve PLHIV (4.6% of asymptomatic patients), routine screening and pre-emptive treatment should be considered as a part of the National Programme in India, which are consistent with the WHO guidelines. In ART-naïve subjects with CD4 ≤ 100 cells/ μl , routine screening has a potential to prevent CM-related morbidity and mortality.

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Institutional and National AIDS Control Organization ethics committee approval was accordingly taken before the initiation of the study.

Conflict of interest

The authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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