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A COMPARATIVE ANALYSIS OF PREVALENCE AND OTHER EPIDEMIOLOGICAL FEATURES OF CRYPTOCOCCAL MENINGITIS IN PRE ART (ANTI-RETROVIRAL THERAPY) AND ART PERIOD – STUDY FROM A TERTIARY CARE CENTRE.

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ABSTRACT **Context & Aim:** With increasing prevalence of HIV infection in India, cases of cryptococcal meningitis increased gradually until the introduction of Antiretroviral Therapy (ART). The study was carried out with the aim of comparing demographic epidemiology of cryptococcal meningitis infection before and after introduction of ART as National Program in general population.

Material and methods: A cross-sectional, observational study was done during years 1997 to 2001 (pre ART period – Group A) and 2008 to 2015 (after ART roll out - Group B). CSF examintion for Cryptococcus spp. of the two groups was done using routine mycological techniques and special culture media. Data of the two groups was compared.

Results: Of the 196 specimens in Group A, 46 had cryptococcal meningitis whereas in Group B, out of 1877 CSF specimens, only 43 were positive. The case reduction was significant ($p < 0.05$). Most cases fell in 20-59 years age group with 2.3% seen in paediatric age in Group B. HIV Positivity was observed in 95.7% cases (Group A) and in 100% cases (Group B). In Group B, 44.1% cases were acute onset as compared with 8.7% in Group A (significant with $p < 0.05$). Seventy seven isolates were identified as *Cryptococcus neoformans*. One was identified as *C. gatti* and the other, serotype D using special media.

Conclusions: Introduction of ART has led to decreased number of cryptococcal meningitis cases but occurrence of rarer species and infection in pediatric age group is noted.

KEYWORDS : antiretroviral therapy, Cryptococcus, meningitis, HIV

INTRODUCTION:

Cryptococcal disease is one of the important opportunistic infection in patients infected with Human Immunodeficiency virus (HIV). In India, once rare, an increasing incidence of this infection was reported with the increasing prevalence of HIV. An increase of upto 15-fold from pre AIDS era to the year 2000 was reported from India (Chakrabarti et al., 2000). The capsular polysaccharide of the fungus is differentiated into 5 serotypes, A, B, C, D and AD (Viviani et al., 2009). Serotype B is *Cryptococcus gatti*. Both of these varieties exist in India (Jain et al., 2005). With the introduction of antiretroviral therapy for HIV infection in India, the incidence of cryptococcosis appears to have been declined in HIV patients. Approximately 70% to 90% of HIV patients have signs and symptoms of meningitis with high mortality rates (Satishchandra et al., 2007; Mitchell et al.,1995).

Our study has included data of cryptococcal meningitis in pre-antiretroviral Therapy (ART) period and the post ART period.

MATERIALS AND METHODS:

A cross-sectional observational epidemiological study of 196 patients clinically suspected of fungal meningitis was carried out in year 1997 to 2001, when the National ART programme was not rolled out in Mumbai.

In the period of 2008 to 2015 when ART was included in the National AIDS Control Programme, 1877 CSF samples from patients suspected clinically of fungal meningitis, submitted for Mycological culture were studied for *Cryptococcus neoformans*. The data of this period was compared retrospectively with the data of 1997 to 2001.

Cerebrospinal fluid (CSF) was aseptically collected. It was processed for direct microscopy by making wet mount, Nigrosin mount, smear for gram stain and culture on Sabouraud's Dextrose Agar and Brain heart infusion agar. CSF which was not turbid, was centrifuged and deposit of CSF was processed in similar way. Cultures of Cryptococcus were identified by urease test, inositol assimilation, nitrate test, L-DOPA and Niger seed agar test (Procop et al., 2006). Serotype identification was done using Canavanine Glycine Bromothymol Blue (CGB) agar (Salkin et al., 1982) and Canavanine Dextrose Bromothymol Blue Thymine (CDBT) agar (Irokanulo et al., 1994). Blue colonies on CGB agar indicated *Cryptococcus gatti*, whereas with *Cryptococcus neoformans*, there was no colour change.

On CDBT agar, colonies without colour change indicated *Cryptococcus neoformans* Serotype A. With blue colonies, it was *Cryptococcus gatti* (Serotype B & C) and Serotype D could be identified by bright orange to red colonies.

For control, following strains obtained from CDC, Atlanta, USA were used.

Serotype A CDC-Y-589-92, Serotype B CDC-Y-586-92, Serotype C CDC-Y-603-92 and Serotype D CDC-Y-600-92

RESULTS:

Of the 196 specimens processed in pre ART period from 1997 to 2001 (Group A), 46 showed growth of *C. neoformans* (23.5%). In the ART era from 2008 to mid 2016 (Group B), out of 1877 CSF specimens from clinically suspected cases of cryptococcal meningitis, 43 cases were positive for cryptococcal meningitis (2.3%). Thus, a markedly decreased prevalence of cryptococcal meningitis was observed in the ART era. The difference is statistically significant with $p < 0.05$. A comparative year wise prevalence of cryptococcal meningitis in these two groups is shown in **Table 1**.

Table1. Year wise distribution of patients with cryptococcal meningitis

Group A (n=196)			Group B (n=1877)		
Year	**No. of Patients with cryptococcal meningitis (n=46)**	**Percentage (100%)**	**Year**	**No. of Patients with cryptococcal meningitis (n=43)**	**Percentage (100%)**
1997	4	8.7	2008	6	13.9

1998	6	13	2009	12	27.9
1999	10	21.7	2010	5	11.6
2000	7	15.3	2011	6	13.9
2001	19	41.3	2012	7	16.3
			2013	4	9.3
			2014	Nil	Nil
			2015	3	7

The average annual incidence is 9.2 cases per annum in Group A and 5.3 cases per annum in Group B. In Group A, highest number of cases were reported in year 2001.

Thereafter however, there was a gradual decline in the cases with the ART becoming freely available in Mumbai under the National AIDS Control Programme from the year 2007-08.The least number of cases were it reported in year 2015 in Group B. There was no seasonal peak observed in both groups as the cases were distributed throughout year.

In Group A, all the patients of cryptococcal meningitis were seen in the age group of 20 to 59 years **(Table 2).**

Table2. Age and sex distribution of the patients with cryptococcal meningitis in the two groups

Group A (n=46)					Group B (n=43)				
Age in years	**Male**	**Female**	**Total**	**Percentage (%)**	**Age in years**	**Male**	**Female**	**Total**	**Percentage (%)**
<1	00	00	00	00	<1	00	00	00	00
1-12	00	00	00	00	1-12	1	00	1	2.3
13-19	00	00	00	00	13-19	00	1	1	2.3
20-39	35	7	42	91.3*	20-39	15	12	27	62.8*
40-59	3	1	4	8.7†	40-59	5	9	14	32.6†
≥60	00	00	00	00	≥60	00	00	00	00
Total	**38**	**08**	**46**	**100**	**Total**	**21**	**22**	**43**	**100**

*P<0.005
†P<0.0005

No case of cryptococcal meningitis was identified in paediatric or geriatric age group.Paediatric patients were encountered only in group B. The male to female ratio in Group A was 4.75:1. There appeared to be a female preponderance of cryptococcal meningitis in the ART period.

In Group A, out of 46 patients with cryptococcal meningitis, 44(95.7%) of the patients were positive for HIV antibodies and 2(4.3%) patients were negative for HIV antibodies. It was not clear if the patients had any other underlying conditions in these two patients, with available investigations. In Group B on the other hand, all the patients were HIV positive.

Commonest symptom found in total 89 patients with cryptococcal meningitis was headache (83.1%), followed by fever (71.9%) and vomiting (66.3%). Altered sensorium (48.3%), convulsions (20.2%), cranial nerve palsy (5.6%) and focal deficit (4.5%) were the other symptoms. The latter two were found only in group A but there was no significant difference in the symptomatology in both the groups.Duration of onset of cryptococcal meningitis in patient groups is shown in **Table 3.**

Table3. Onset of symptoms in patients with cryptococcal meningitis in the two groups

Onset of Meningitis	Group A (n=46)	Percentage (%)	Group B (n=43)	Percentage (%)
Acute (≤ 2 days)	04	8.7‡	19	44.1‡
Subacute (3-7 days)	10	21.7	15	35.7
Chronic >7days)	32	69.6	9	21.4
Total	**46**	**100**	**43**	**100**

‡P value - <0.0005

In Group B, the presentation of meningitis was acute in 44.1% which was much more than was seen in Group A (8.7%). Tuberculosis was the common co-infection seen in the patients with cryptococcal meningitis. The association of active tuberculosis was seen marginally increased in Group B (Table 4).

Table4. Coinfection with tuberculosis in patients with cryptococcal meningitis in the two groups

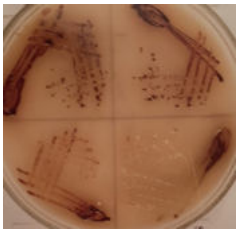
		Group A (n=46)		Group B (n=43)	
		No.	**%**	**No.**	**%**
Active TB	Pulmonary	10	21.7	9	20.9
	Abdominal	01	2.2	2	4.6
	Lymphnode	00	00	2	4.6
	Miliary	00	00	1	2.3
Total		**11**	**24**	**14**	**32.4**
Past History of TB	Pulmonary	08	17.4	02	4.6
	Lymphnode	01	2.2	00	00
Total		**09**	**19.6**	**02**	**4.6**

All the patients diagnosed with cryptococcal meningitis were treated with amphotericin B in recommended dosages. Out of the 46 patients in Group A, 27 (58.7%) died, 10 (21.7%) were discharged and 09(19.6%) left against medical advice whereas, among Group B patients, 22 (51.2%) were discharged, 14 (32.5%) died and 07 (16.3%) left against medical advice. The mortality rate was found decreased by 26.2% probably due to early reporting by patients and timely institution of therapy, a result of improved awareness.

In Group A, 46 CSF samples were positive for capsulated yeasts on direct examination. Out of these 46 specimens, 45 grew *Cryptococcus neoformans* on culture but 1 did not show any growth on Sabouraud's dextrose agar despite patient not being on antifungal therapy. In Group B, 43 CSF samples were positive for capsulated yeasts on direct examination. However, out of these 43 CSF specimens, 29 grew *Cryptococcus species* on culture, 12 specimens did not show any growth on Sabouraud's dextrose agar. Amongst them, 8 patients were being treated with amphoterecin B, 2 with fluconazole and 2 patients were not on any antifungal therapy but their CSF specimens grew contaminants.

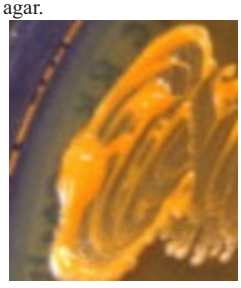
All the Cryptococcus isolates were tested on bird seed agar. They showed dark brown pigmented colonies except one isolate from a patient in group A, which showed light brown pigmentation (hypomelanotic) **(Figure 1)** and this isolate was identified as *Cryptococcus gatti* when tested on CGB agar**.**

Figure1. Brown pigmented colonies of *Cryptococcus neoformans* on Niger seed agar. Note the weak brown colored colonies of *C. gatti* in comparison with others.



In group B, one isolate was identified as *Cryptococcus neoformans* serotype D on CDBT agar **(Figure 2).**

Figure2. Orange coloured colonies of *Cryptococcus neoformans* serotype D on CDBT agar.



All the other isolates were identified as *Cryptococcus neoformans* as tested on CGB and CDBT media.

DISCUSSION:

The overall prevalence of cryptococcal meningitis, among patients clinically suspected of fungal meningitis in Group A was higher than in Group B as Group B fell in ART era. In Group A, over a period of 5 years, an increasing prevalence of cryptococcosis has been encountered. The decreasing incidence of cryptococcosis in the period

from 2008 to 2015 can be extrapolated in the light of a parallel decrease in the incidence of HIV infection. In a study, the number of new HIV infections were found to have reduced substantially in Mumbai during the period 2001 to 2010 (Mishra et al.,2012). An increasing incidence of cryptococcal meningitis in pre-ART period has also been reported by Chakrabarti et al (2000).They have reported an average annual incidence of 11.6 cases/ annum which is in comparison with the 9.2 cases/ annum reported in Group A in our study.

A decrease in incidence of cryptococcal disease was seen after introduction of effective ART in Europe and North America, where even hospitalisations were reduced to half (Pyrgos et al.). Incidence of cryptococcal meningitis is also found to be reduced from a peak between 2005 and 2009 in South Africa (Rapid Germs South Africa Annual Report, 2014)

As opposed to Group A, in Group B, cases are seen in all age groups and especially in the older age group of 40 t0 59 years, owing probably to longevity offered by ART. In a review of 32 years of experience with cryptococcal meningitis, it has been reported that the majority of cases occur between the ages of 20 and 50 years (DeWytt et al., 1982). Studies in recent years depict high incidence of cryptococcal meningitis in patients between 20 and 40 years of age (Lucia et al., 2013; Rapid Germs South Africa Annual Report, 2014). A study from Brazil showed that 69.8% of cases occurred in age group 30-49 (Joslaine st al., 2018). Cryptococcosis is uncommon in children (Sow et al., 1998). In 17 years study period at a Brazilian state, only 2.3% of cryptococcal meningitis cases were recorded in pediatric population (Joslaine st al., 2018).

In a review on cryptococcosis in India by Banerjee U et al. in 2001, out of 118 cases reported by different authors, 78% of patients with cryptococcosis are in the age group of 20-39 years and 9.3% between 40 to 59 years.Paediatric cases constituted 1.7%. In 106 patients of cryptococcosis reported by Chuck and Sande, in 1989, the range of age was 23-65 years with a mean of 38. Some populations may be at higher risk for cryptococcosis on the basis of increased exposure to infectious particle or on genetic susceptibility. A suggestion that human genetic factors could confer susceptibility to *Cryptococcus neoformans* is indicated by the description of cryptococcosis in two siblings who had no obvious predisposing conditions for the infection (Sow et al., 1998). Numerous studies have reported a predominance of male patients among individuals with cryptococcosis, both before the HIV epidemic and afterwards, probably related to outdoor activities (Sow et al., 1998; Mcclelland et al., 2013). Anuradha et al. (2015) mentioned that in their study of cryptococcal meningitis in patients living with HIV, 36% of subjects were unskilled workers. In our Group B, female preponderance is seen. Chakrabarti et al. (1995) have reported a preponderance of males, 4.0:1.4. Reports from other parts of the country and abroad also indicate a male predominance (Chakrabarti et al., 2000; Mitchell et al., 1995; Lucia et al.,2013; Duggal et al., 2014; Chakrabarti et al., 1995; Padhye et al., 1993). Manoharan G et al. (2001) however report a male to female ratio of 14.5:1.

Diethylstilbesterol has been found to inhibit the growth of *C. neoformans* in vitro. Furthermore, male guinea pigs appear to be more susceptible to cryptococcal infection than females (Dash et al., 2014). Testosterone seems to induce greater capsule production and lower macrophage efficiency (Kalra et al., 1999). Localization of infection in the prostate gland could be another factor contributing to greater frequency of cryptococcal infection in men.

Since 1981, the most common risk factor for cryptococcosis has been HIV infection (Sow et al., 1998). In Group B, all patients had HIV as the predisposing factors. Khanna et al. from South India in 1996, assessed 60 patients with cryptococcal infection of the CNS. They have reported HIV as the predisposing factor in 30% of patients (Khanna et al., 1996). Hashimoto et al. in 2013 also reported that majority of the patients (85.5%) in their study had AIDS. Prevalence noted in other studies ranges from 2.24% to 94.6% among people living with HIV and 1.7% in all hospitalized patients (range 0.9% to 26.8%) (Chakrabarti et al., 2000; Duggal et al., 2014; Kalra et al., 1999; Millogo et al., 2004).

No predisposing factors could be identified in as high as 23.4% to 40% of the cases studied by some workers in India. These authors have reported HIV as a predisposing factor in 57.4% and 30% respectively (Chakrabarti et al., 2000; Khanna et al., 1996).

All patients with acute manifestations in Group A were HIV positive.In Group A, more patients showed chronic duration of symptoms. Whereas in Group B, subacute duration was more common. Several studies have shown mean duration of symptoms before diagnosis and the hospital stay to be 27.71 days (2-121 days) (Millogo et al., 2004).

Considering the high rate of HIV positivity in our patients with cryptococcal meningitis, a high rate of co-existent tuberculosis was expected. A significantly large number of patients with cryptococcal meningitis in Group A gave a past history of tuberculosis in contrast with Group B. Anuradha et al. (2015) reported 32% coexistent pulmonary tuberculosis cases and 12% extrapulmonary tuberculosis cases with cryptococcal meningitis in PLHIV.

Coexistent pulmonary tuberculosis and extrapulmonary tuberculosis was seen in 34.7% and 8.6% cases of cryptococcal meningitis associated with HIV in a study by Duggal et al. (2014). Thakur et al. (2008) reported 15.3% patients in their study had coexistent pulmonary tuberculosis.

More than half patients succumbed to cryptococcal meningitis in Group A, reason could be due to unavailability of ART leading to loss of cellular immunity. Chakrabarti et al. in 2000, in another study, reported a mortality rate of 20.4% and 48.7% patients improved and were discharged. In their study group, the HIV positivity of patients with cryptococcal meningitis was 57.4% which was much lower in comparison with patients in Group A with HIV positivity of 95.7% (Table 3). Kalra S et al. in 1999 reported a mortality rate of 40% among the 15 patients who were HIV positive, diagnosed as cryptococcal meningitis and 46.7% patients responded to therapy. Mortality was also reported to be heavy with 45.9% of deaths (range 42.2% to 71.1%), more than half of the patients died in most studies (Mcclelland et al., 2013; Millogo et al., 2004).

However, more than half of the patients with cryptococcal meningitis improved on treatment and were discharged. Naik et al. (2017) reported a mortality rate of 12.3% whereas favourable outcome was seen in 74.2% of patients. Duggal et al. (2014) reported mortality of 40% in HIV associated cryptococcal meningitis patients undergoing treatment with amphoterecin B and Flucytosine, within first two weeks. Whereas, Chuck and Sande (1989) have reported a mortality rate of 19.1% among patients with cryptococcal meningitis.

The India ink examination is a particularly useful and rapid diagnostic test for cryptococcal meningitis. Assogba et al. (2015) observed that direct examination by India Ink was positive in 90.88% of CSF specimens and culture was positive on Sabouraud's dextrose medium in 98.4%. In India, Manoharan G et al. (2001) have reported a very low sensitivity of 35.5% even if the patients were HIV positive. Khanna et al. (1996) have reported a sensitivity of 80%. Duggal et al. (2014) reported sensitivity of 98% by India Ink Examination.

In our study, microscopy was positive in 89 patients (100%). A similar finding was reported by Chakrabarti A et al. in 2000. In their study, of the 58 India ink positive patients only 1 was negative on culture. However, Anuradha et al. (2015) reported that 64% cases were positive by culture whereas only 60% were positive on India ink examination. High culture positivity was indicative of high fungal burden in their patients.There are two clinical situations when culture of Cryptococci may be difficult. Either it may be due to low burden of organisms or in patients who have received antifungals.

In our study *C. gatti* was isolted from one patient in Group A. Our findings were similar to that of Mcclelland EE et al.,(2013).

Duggal et al. (2014) in their study, identified all strains as *Cryptococcus neoformans* var *grubii* by PCR fingerprinting analysis. Nyazika et al. (2016) identified 4 strains as *C. neoformans* and 1 as *C. gattii* from five pediatric HIV associated cryptococcal meningitis cases. Padhye et al. (1993) examined 18 clinical isolates of *Cryptococcus neoformans* from India out of which three were *Cryptococcus neoformans* var *gattii* (serotype B) and all others were *C. neoformans* var. *neoformans*. Among 199 cases of cryptococcal infections, Mukhopadhyay et al. (2017) reorted 10% infections caused by *C. gattii*.

Progress in the understanding of cryptococcosis is far from satisfactory. It still remains one of the most important and potentially

fatal mycotic disease affecting the immunocompromised. The behaviour of the disease in the scenario of specific and supportive treatments and the evolution of the disease in various time frames is important in understanding its epidemiology.

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