Detection of *Cryptococcus* by conventional, serological and molecular methods

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The rising incidence of cryptococcosis in India is posing a serious threat. Due to lack of sensitive methods for diagnosis, high morbidity and mortality are associated with the disease. Early diagnosis is essential to prevent serious complications. Therefore, we attempted to find highly sensitive and specific detection methods. A comparative evaluation of the detection of cryptococcosis was done by conventional (direct microscopy and culture) and rapid diagnostic [latex agglutination test (LAT), enzyme immunoassay (EIA) and PCR] methods. The study was done on 359 samples from 52 positive patients and 30 negative controls in an Indian set-up. Evaluation was done for cerebrospinal fluid (CSF), serum and urine separately. The diagnostic value of the tests was assessed in pre-treatment samples, and follow-up tests were also done on samples obtained after initiation of treatment. PCR had the highest sensitivity, followed by EIA and LAT, both before and after treatment. The positive detection by LAT, EIA and PCR was the longest in CSF (>90 days), followed by serum (~65 days) then urine (~45 days) after initiation of treatment. Our results indicated that the sensitivity and specificity of PCR and EIA were comparable in urine, CSF and serum for diagnosis of cryptococcosis.

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INTRODUCTION

Cryptococcus neoformans causes pulmonary infection and meningoencephalitis. The rising incidence of cryptococcosis in India is posing a serious threat (Banerjee *et al.*, 1994, 2001, 2004; Banerjee, 2005; Chakrabarti & Gupta, 1997; Chakrabarti *et al.*, 2000). With the onset of AIDS, its occurrence has escalated manyfold (Currie & Casadevall, 1994). Early diagnosis and institution of specific antifungal therapy are imperative to minimize the severity of infection. The laboratory diagnosis of cryptococcosis is based on direct demonstration, culture, and antigen detection by latex agglutination test (LAT). Microscopic methods and culture, though specific, show a sensitivity of 50–80% (Snow & Dismukes, 1975). Also, culture takes time and requires more labour and large volumes of samples. LAT is more sensitive but suffers from the limitation of false positivity (Boom *et al.*, 1985; Heelan *et al.*, 1991; Kornfeld & Worthington, 1980; MacKinnon *et al.*, 1978; Millon *et al.*, 1995; Sachs *et al.*, 1991; Stoeckli & Burman, 2001; Whittier *et al.*, 1994) as well as high rates of false negativity (Currie *et al.*, 1993; Stamm & Polt, 1980; Coovadia & Solwa, 1987). The apparent

subjectivity of reading and grading the LAT reaction poses additional problems, especially in borderline cases. Enzyme immunoassay (EIA) is another method for detection of *C. neoformans* capsular polysaccharide antigens in clinical samples. Studies have demonstrated the low cross-reactivity of this EIA (Casadevall *et al.*, 1992; Frank *et al.*, 1993; Scott *et al.*, 1981; Sekhon *et al.*, 1993). PCR offers an excellent alternative for the early diagnosis of cryptococcosis compared to conventional methods, as it is rapid, can detect low fungal load, and can be used for a small sample size (Paschoal *et al.*, 2004; Imwidthaya & Pongvarin, 2000). Here, we have carried out a comparative study of conventional methods, LAT, EIA and PCR on a total of 359 samples from 82 patients. The comparative evaluation was done on cerebrospinal fluid (CSF), serum and urine samples separately.

METHODS

Standardization of diagnostic test systems. Normal saline and negative CSF, serum and urine were spiked with 10^3 – 10^1 cell ml⁻¹ of *Cryptococcus neoformans* (ATCC 24067 and clinical isolates), to establish the sensitivity of different test systems. For serum, only LAT, EIA and PCR were performed as per the standard protocol, while CSF and urine were tested by culture, direct microscopy, as well as by LAT,

Abbreviations: CSF, cerebrospinal fluid; EIA, enzyme immunoassay; LAT, latex agglutination test.

EIA and PCR. The same methodology was applied to spiking with *Candida albicans*, to test for the specificity.

Patients and samples. A total of 359 samples from 82 patients with suspected cases of cryptococcal meningitis were collected from September 2004 to October 2007 and investigated at the mycology laboratory of the All India Institute of Medical Sciences. Consent had been obtained from the patients prior to investigation and IRB approval was obtained for the study. The samples were tested by culture, microscopy, LAT, EIA and PCR.

A total of 269 samples from 52 of 82 patients were detected as positive for *Cryptococcus*. Among these, those obtained within a day of diagnosis of infection and considered pre-treatment samples included 46 CSF samples, 35 serum samples and 28 urine samples. Follow-up samples obtained after the initiation of treatment included 50 CSF samples, 46 serum samples and 64 urine samples. Thirty patients who were negative for cryptococcosis but presented with other forms of chronic meningitis [tuberculous meningitis (TBM; $n=28$); neurosyphilis ($n=1$); TBM with herpes simplex virus ($n=1$)] were taken as controls. The samples collected from the control group were 30 samples each of CSF, serum and urine. The CSF, serum and urine obtained from positive patients prior to treatment and from controls were used for assessing the diagnostic value of the tests; the follow-up tests were also performed on samples obtained after initiation of the treatment.

Clinical data. Among 52 cryptococcal positive cases, 31 patients were HIV-positive and 21 were HIV-sero-negative. All HIV-positive patients had CD4⁺ T-cell counts <200 cells μl^{-1} . The demographic and clinical data including mortality were collected from these patients. Of the patients who died, 32 % ($n=10$) were HIV-positive and 43 % ($n=9$) were HIV-negative. The clinical characteristics of *Cryptococcus*-positive patients are shown in Table 1 and the mortality data are described in Table 2.

Processing of samples. The standard method was followed for the processing of samples (Ajello *et al.*, 1963). CSF and urine were centrifuged at 1000 *g* for 15 min. The pellet was used for investigation by culture, direct microscopy (India ink wet mount, Gram stain) and PCR, while the supernatant was tested by LAT and EIA. All the samples were tested without dilution. CSF was cultured on brain heart infusion agar with gentamicin (26 $\mu\text{g ml}^{-1}$). Culture tubes were incubated at 37 and 30 °C. Urine samples were inoculated in Sabouraud dextrose agar tubes with gentamicin (26 $\mu\text{g ml}^{-1}$). Culture tubes were incubated at 37, 30 and 25 °C. The tubes were screened daily for the presence of the organism and discarded after 1

month if there was no growth. The organism generally grew in 3–5 days. Clinical isolates were identified on the basis of urease production, lack of hyphae on cornmeal agar medium, growth at 37 °C and sensitivity to 0.5 μg cycloheximide ml^{-1} . Variety differentiation was done on glycine-cycloheximide-phenol red (GCP) medium. Tubes were incubated for up to 5 days at 25 °C for observation of growth and change in the colour of the indicator. Serotyping was not done to distinguish between the structures of the capsular polysaccharide, since the present study was focused on finding a sensitive and specific detection system for the rapid diagnosis of cryptococcosis. All the tests were standardized with fresh clinical isolates. The isolates were maintained in 80 % glycerol stocks and stored at –80 °C for future studies.

Blood was centrifuged at 1000 r.p.m. for 5 min to obtain serum (Ajello *et al.*, 1963) and subjected to serological (LAT, EIA) and molecular (PCR) methods of detection only.

LAT assays. Samples were centrifuged and the supernatant was used for the latex test for *C. neoformans* antigen detection. The test was performed using the CALAS kit (Meridian Bioscience), according to the manufacturer's instructions. Boiling CSF and urine for 5 min and then cooling them to room temperature prior to testing limited non-specific interference. Serum specimens were treated with Pronase prior to testing by LAT.

EIA. A screening assay was performed as per the manufacturer's instructions with the PREMIER Cryptococcal Antigen EIA (Meridian Bioscience). The A_{450} was determined by spectrophotometry. The positive and negative cut-off values were 0.150 and 0.10, respectively. Indeterminate results (values between 0.1 and 0.15) were repeated.

Template DNA preparation. DNA was extracted from all samples using the QIAamp tissue kit (Qiagen), with minor modifications to the manufacturer's instructions, especially for lysis. Samples were boiled for 5 min and centrifuged to obtain 200 μl pellet. This was then treated with 500 μl each of 6 M guanidine thiocyanate and phenol saturated in Tris (Mitchell *et al.*, 1994). The entire mix was boiled for 15 min and then centrifuged. Two hundred microlitres of spheroplasts was incubated with 180 μl ATL buffer and proteinase K at a final concentration of 1 mg ml^{-1} at 56 °C for 30 min. After centrifugation and washing, total DNA was eluted with 50 μl warm AE buffer and stored at –20 °C for further use.

Primers and PCR. Oligonucleotides Fungus (Fun) I (GTT AAA AAG CTC GTA GTT G) and Fungus II (TCC CTA GTC GGC ATA GTT TA), which are complementary to highly conserved regions within

Table 1. Clinical characteristics of *Cryptococcus*-positive study patients ($n=52$)

TB, Tuberculosis; TBM, tubercular meningitis; SLE, systemic lupus erythematosus; PRT, post-renal transplant; CLL, chronic lymphocytic leukaemia.

	HIV-positive	HIV-negative
<i>n</i>	31	21
Median age (range)	33 (13–53) years	38 (20–65) years
Male to female ratio	9.3:1	1.3:1
Form of disease	29 (93.5 %) meningitis; 1 (3 %) fungaemia; 1 (3 %) cryptococcal lymphadenitis	16 (76 %) meningitis; 1 (5 %) skin; 1 (5 %) ulcer; 1 (5 %) wound infection; 2 (10 %) fungaemia
Mortality	10 (32.3 %)	9 (43 %)
Underlying disorders	TB, 20 (64 %) (pulmonary 7; disseminated 8; tuberculous lymphadenitis 1; TBM 4); SLE, 1 (3 %); oral thrush, 1 (3 %); none, 9 (29 %)	PRT, 8 (38 %); TB, 5 (24 %) (pulmonary 2; TBM 3); CLL, 1 (5 %); SLE, 1 (5 %); sarcoidosis, 1 (5 %); lupus nephritis, 1 (5 %); <i>Nocardia</i> wound infection, 1 (5 %); none, 3 (14 %)

Table 2. Mortality table of cryptococcosis-positive study patients

Death day is counted from the day of admission to the hospital to the day when the patient expired.

HIV-positive	Death day	Underlying disorder	HIV-negative	Death day	Underlying disorder
Patient 1	3	Pulmonary tuberculosis	Patient 11	6	Tubercular meningitis
Patient 2	14	Disseminated tuberculosis	Patient 12	2	Chronic lymphocytic leukaemia
Patient 3	6	None	Patient 13	2	Chronic renal failure, post-renal transplant
Patient 4	18	Systemic lupus erythematosus	Patient 14	26	Post-renal transplant
Patient 5	40	Tubercular meningitis	Patient 15	53	Varicella, disseminated tuberculosis
Patient 6	2	None	Patient 16	11	Post-renal transplant, pneumonia
Patient 7	32	Pulmonary tuberculosis	Patient 17	11	Lupus nephritis – stage 4
Patient 8	18	None	Patient 18	15	Post-renal transplant
Patient 9	18	None	Patient 19	65	None
Patient 10	59	Tubercular meningitis			

18S rDNA of several pathogenic fungi (including *C. neoformans*), generate a 429 bp amplicon. Cryp I (TCC TCA CGG AGT GCA CTG TCT TG) and Cryp II (CAG TTG TTG GTC TTC CGT CAA TCT A), which are nested primers for Fungus/II, amplify a 278 bp region within 18S rDNA specific for *C. neoformans* (Bialek *et al.*, 2002). Amplification was performed in 50 µl with a 20 µl suspension of DNA, 0.4 mM dNTPs, 3 U *Taq*, 5 µl 10 × reaction buffer (10 mM Tris/HCl, pH 9.0; 50 mM KCl; 0.1 % Triton X-100) and 1 µM of each primer. An identical mix was made for nested PCR except that 1 µM each primer Cryp I and II was used, and 1 µl Fungus/II amplification mixture was used as template. After initial denaturation at 94 °C for 5 min in the first PCR, the 35-cycle amplification profile consisted of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. Final elongation was at 72 °C for 5 min. For nested PCR, the initial denaturation was at 94 °C for 5 min, followed by a 30-cycle two-step amplification profile: 94 °C for 30 s and 72 °C for 1 min. Final elongation was at 72 °C. PCR products were electrophoretically analysed on 1 % agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator (Bialek *et al.*, 2002).

Statistical analysis. The sensitivity and specificity of test systems were calculated using SPSS 15 software on CSF, serum and urine obtained before antifungal treatment and negative controls. Culture was considered the gold standard.

RESULTS AND DISCUSSION

Identification of *C. neoformans* in clinical samples

All the isolates were identified as *C. neoformans* var. *neoformans*. None of the isolates belonged to *C. neoformans* var. *gattii*, as per the variety differentiation test on GCP medium.

Standardization of test systems

India ink and culture detected 100 cells ml⁻¹, while Gram stain, LAT, EIA and PCR could detect 1000, 50, 20 and 10 cells ml⁻¹, respectively, in normal saline and negative CSF and urine spiked with *C. neoformans*. Spiked serum samples were tested by LAT, EIA and PCR and the sensitivity was comparable. PCR, EIA and LAT were

negative with *Candida albicans*. Experiments for standardization and comparative evaluation of the tests were done in triplicate, and the same results were obtained.

Diagnostic value of tests with pre-treatment samples

Culture, Gram stain, India ink, LAT, EIA and PCR were performed on CSF, serum and urine separately and compared to estimate sensitivity and specificity.

The test results are given below.

CSF. The sensitivity and specificity of different tests are presented in Table 3. PCR and EIA showed a 100 % sensitivity and specificity. Culture and India ink results were almost on a par. LAT had 100 % sensitivity but gave false-positive results for four samples, due to the presence of Gram-negative bacilli ($n=3$, LAT titre 1:2) and *Trichosporon* ($n=1$, LAT titre 1:4).

Serum. The sensitivity and specificity are shown in Table 4. The sensitivity of LAT, EIA and PCR was similar; however, the specificity of LAT decreased due to false-positivity in two samples (LAT titre 1:2).

Urine. Culture, Gram stain and India ink were positive in 21, 18 and 32 % of samples, respectively, while LAT, EIA and PCR were positive in all the samples (100 %) before treatment. Table 5 depicts the sensitivity and specificity values and false-positivity for LAT in three samples (LAT titre 1:2; Gram-negative bacilli).

We found that the specificity of LAT decreased due to false-positivity in a total of 2.5 % ($n=9$; HIV-negative). Conversely, EIA and PCR showed no cross-reactivity. Engler & Shea (1994) demonstrated that EIA did not cross-react with specimens whereas LAT did. Our previous studies also demonstrated a false-positivity of 6.3 % for LAT (Saha *et al.*, 2008). PCR has been shown to be very specific too (Paschoal *et al.*, 2004; Rappelli *et al.*, 1998;

Table 3. Sensitivity and specificity of different diagnostic tests in CSF ($n=76$)

PPV, Positive predictive value; NPV, negative predictive value.

Test		Diseased ($n=46$)	Control ($n=30$)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Agreement (%)
Culture	Positive	44	0	95.7 (84, 99.2)	100 (85.9, 100)	100 (90, 100)	93.8 (77.8, 98.9)	97.4
	Negative	2	30					
India ink	Positive	43	0	93.5 (81.1, 98.3)	100 (85.9, 100)	100 (89.8, 100)	90.9 (74.5, 97.6)	96
	Negative	3	30					
Gram stain	Positive	39	0	84.8 (70.5, 93.2)	100 (85.9, 100)	100 (88.8, 100)	81.1 (64.3, 91.4)	90.8
	Negative	7	30					
LAT	Positive	46	4	100 (90.4, 100)	86.7 (68.4, 95.6)	92 (79.9, 97.4)	100 (84, 100)	94.7
	Negative	0	26					
EIA	Positive	46	0	100 (90.4, 100)	100 (85.9, 100)	100 (90.4, 100)	100 (85.9, 100)	100
	Negative	0	30					
PCR	Positive	46	0	100 (90.4, 100)	100 (85.9, 100)	100 (90.4, 100)	100 (85.9, 100)	100
	Negative	0	30					

Table 4. Sensitivity and specificity of different diagnostic tests in serum ($n=65$)

PPV, Positive predictive value; NPV, negative predictive value.

Test		Diseased ($n=35$)	Control ($n=30$)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Agreement (%)
LAT	Positive	35	2	100 (87.7, 100)	93.3 (78.3, 98.4)	94.6 (80, 98.9)	100 (84.5, 100)	96.9
	Negative	0	28					
EIA	Positive	35	0	100 (87.7, 100)	100 (85.9, 100)	100 (87.7, 100)	100 (85.9, 100)	100
	Negative	0	30					
PCR	Positive	35	0	100 (87.7, 100)	100 (85.9, 100)	100 (87.7, 100)	100 (85.9, 100)	100
	Negative	0	30					

Table 5. Sensitivity and specificity of different diagnostic tests in urine ($n=58$)

PPV, Positive predictive value; NPV, negative predictive value.

Test		Diseased ($n=28$)	Control ($n=30$)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Agreement (%)
Culture	Positive	6	0	21.4 (9, 41.5)	100 (85.9, 100)	100 (51.7, 100)	57.7 (43.3, 71)	62.1
	Negative	22	30					
India ink	Positive	9	0	32.1 (16.6, 52.4)	100 (85.9, 100)	100 (82.9, 100)	61.2 (46.2, 74.5)	67.2
	Negative	19	30					
Gram stain	Positive	5	0	17.9 (6.8, 37.6)	100 (85.9, 100)	100 (46.3, 100)	56.6 (42.4, 69.9)	60.3
	Negative	23	30					
LAT	Positive	28	3	100 (85, 100)	90 (72.3, 97.4)	90.3 (73.1, 97.5)	100 (84.5, 100)	94.8
	Negative	0	27					
EIA	Positive	28	0	100 (85, 100)	100 (85.9, 100)	100 (85, 100)	100 (85.9, 100)	100
	Negative	0	30					
PCR	Positive	28	0	100 (85, 100)	100 (85.9, 100)	100 (85, 100)	100 (85.9, 100)	100
	Negative	0	30					

Bialek *et al.*, 2002). Hence LAT is less specific than EIA and PCR.

In pre-treatment samples, our results showed that, with conventional methods, CSF was the best sample for the detection of cryptococcosis and urine may not be very good. However, urine proved to be a good quality sample for detection by LAT, EIA and PCR, as these tests were highly sensitive. Urine, being a non-invasive sample and easily accessible, may be used for detection of *Cryptococcus* by these methods. CSF and serum were also equally good for the detection of cryptococcosis by LAT, EIA and PCR.

Follow-up test results

Various tests such as direct microscopy, culture, LAT, EIA and PCR were performed on follow-up samples. This could give some insight regarding prolongation of the activity of the disease and response to treatment in follow-up patients. Samples were obtained depending on the condition of the patient at the discretion of the clinician. We observed a different positivity pattern in follow-up samples. Variations in the test pattern were observed over specific intervals of time (ranging from 6 to 90 days) after initiation of the treatment for CSF, serum and urine as described in Table 6 and Fig. 1. The results are summarized below.

CSF. The overall percentage positivity of CSF before treatment ranged from 84 to 100%: 96% by culture, 85% by Gram stain, 94% by India ink and 100% by LAT, EIA and PCR. However, after the initiation of treatment, the positivity decreased considerably for direct demonstration

(62% for Gram stain and 64% for India ink) and culture (42%) but not substantially for serological methods (92% for LAT and EIA) and PCR (96%). Specimens had LAT titres ranging from $\geq 1:1024$ (6–13 days) to $\geq 1:64$ (30–37 days) and $\leq 1:4$ (66–90 days). There were 29 culture-negative samples after treatment. Among them, the Gram stain was positive in 34.5% ($n=10$), India ink in 38% ($n=11$), LAT and EIA in 86.2% ($n=25$) and PCR in 93% ($n=27$).

Serum. The overall positivity in serum was 100% by all methods of detection before treatment but it decreased to 94% for LAT, EIA and PCR after initiation of therapy. The LAT titre ranged from $\geq 1:2048$ (6–13 days), to $\leq 1:16$ (30–37 days), to $\leq 1:2$ (46–65 days), to negative (66–90 days).

Urine. The positivity in urine was lower before therapy compared to CSF and serum by standard methods (21% by culture, 18% by Gram stain and 32% by India ink). Also, there was a notably lower positivity after initiation of therapy (6% by culture, 0% by Gram stain and 5% by India ink). However, there was not much difference by LAT, EIA and PCR, which demonstrated a reduction to 84, 89 and 92%, respectively. Specimens had LAT titres ranging from $\geq 1:64$ (6–13 days), to $\leq 1:2$ (30–37 days) to negative (66–90 days). Among 60 culture-negative samples, following treatment 83.3% ($n=50$) were positive by LAT, 88.3% ($n=53$) by EIA and 92% ($n=55$) by PCR. Our study detected an EIA+/LAT– result in three urine samples, where patients had been on treatment for at least 3 weeks. Thus EIA could be positive

Table 6. Variation of tests over specific intervals of time after beginning antifungal therapy (number of positive samples/total samples)

		Days after starting antifungal treatment (AT)							
		6–13	14–21	22–29	30–37	38–45	46–65	66–90	Total (AT)
CSF									
Culture	44/46	6/7	5/10	5/9	4/6	1/9	0/4	0/5	21/50
Gram stain	39/46	6/7	7/10	7/9	4/6	4/9	2/4	1/5	31/50
India ink	43/46	7/7	7/10	6/9	5/6	4/9	2/4	1/5	32/50
LAT	46/46	7/7	10/10	9/9	6/6	9/9	2/4	3/5	46/50
EIA	46/46	7/7	10/10	9/9	6/6	9/9	2/4	3/5	46/50
PCR	46/46	7/7	10/10	9/9	6/6	9/9	4/4	3/5	48/50
Serum									
LAT	35/35	23/23	9/9	6/6	3/3	1/1	1/2	0/2	43/46
EIA	35/35	23/23	9/9	6/6	3/3	1/1	1/2	0/2	43/46
PCR	35/35	23/23	9/9	6/6	3/3	1/1	1/2	0/2	43/46
Urine									
Culture	6/28	4/29	0/14	0/6	0/7	0/2	0/4	0/2	4/64
Gram stain	5/28	0/29	0/14	0/6	0/7	0/2	0/4	0/2	0/64
India ink	9/28	3/29	0/14	0/6	0/7	0/2	0/4	0/2	3/64
LAT	28/28	29/29	14/14	5/6	5/7	0/2	1/4	0/2	54/64
EIA	28/28	29/29	14/14	6/6	6/7	1/2	1/4	0/2	57/64
PCR	28/28	29/29	14/14	6/6	6/7	2/2	2/4	0/2	59/64

Fig. 1. Percentage positivity of various tests over specific intervals of time (ranging from 6 to 90 days) after beginning antifungal therapy for CSF, serum and urine. BT denotes percentage positivity before treatment.

for an extended period. This compares favourably with findings demonstrating elevated EIA titres continuing longer into the treatment period than those by LAT (Frank *et al.*, 1993; Scott *et al.*, 1980). Gade *et al.* (1991) also highlighted the higher sensitivity of EIA in contrast to LAT and detected EIA +/LAT- in eight samples.

The detection of *C. neoformans* DNA in clinical specimens by PCR has been studied by a small number of groups (Rappelli *et al.*, 1998; Bialek *et al.*, 2002; Evertsson *et al.*, 2000; Mitchell *et al.*, 1994; Prariyachatigul *et al.*, 1996). Bialek *et al.* (2002) concluded that a nested PCR assay could be broadly applicable. Our PCR results compared favourably with the reported detection limits of 10 cells ml^{-1} and could be used for regular routine diagnosis because of the high sensitivity.

There was 100 % concordance between EIA and PCR in all the samples before treatment, demonstrating that both tests are comparable. When EIA was negative, all other tests were also negative post-treatment, except PCR. Positive results were obtained with PCR in two CSF samples from the same patient after 8 weeks of treatment and with two urine samples from different patients after 4 weeks of treatment, while EIA tested negative in these. This establishes PCR as a more sensitive test. Also, PCR did not detect DNA in any of the control samples, dem-

onstrating its specificity. This was due to the high specificity of the nested primers for *C. neoformans*. Paschoal *et al.* (2004) found PCR sensitivity and specificity to be the highest (92.9 % and 100 %, respectively), followed by India ink (85.7 % and 100 %, respectively) and culture (76.8 % and 100 %, respectively). In our study too, PCR and serological tests exhibited the highest sensitivity. In a study by Rappelli *et al.* (1998), nested PCR was negative in specimens from patients with bacterial and viral meningitis, a finding that correlates well with the current study. Thus PCR, being a more sensitive and specific test, can be introduced on a regular basis for diagnosis. The sensitivity of LAT, EIA and PCR is comparable. However, EIA proved to be better than LAT due to its clear cut-off, the absence of the requirement for specimen pre-treatment and its high specificity (Gordon & Vedder, 1966; Illnait *et al.*, 2001; Gade *et al.*, 1991). Thus EIA can be used as a serological diagnostic test in adjunct to PCR.

For samples obtained after the initiation of treatment (follow-up samples), culture was positive in CSF for about 4–6 weeks and direct microscopy for 9–10 weeks, while urine positivity by conventional techniques persisted only for about a week. The duration of positivity of LAT, EIA and PCR was longest in CSF (>90 days), followed by serum (~65 days) then urine (~45 days). Although the fungal load in urine was low, the presence of cryptococcal

antigen in urine was sufficient to be detected by serological methods. Accordingly, detection in urine, especially by PCR and EIA, may be helpful when obtaining invasive samples such as CSF and serum proves difficult.

Thus, in conclusion, direct microscopy (India ink, Gram stain), culture, serological methods (LAT and EIA) and a molecular method (PCR) were done on 359 samples. We demonstrated that LAT, EIA and PCR have a higher sensitivity than direct microscopic methods. These were positive even when culture was negative in samples of low inoculum or in patients on antifungal treatment. Although test positivity diminished with treatment for each patient, their response to treatment over a time interval differed due to variations in the initial fungal load and severity of disease. Thus only samples preceding treatment were considered for evaluation of sensitivity and specificity and only the positivity pattern was observed in follow-up samples. There was no difference in the detection of *Cryptococcus* by various tests, irrespective of the HIV status. However, the persistence of infection was longer in HIV-positive individuals. CSF culture was positive for about 3–4 weeks in HIV-positive patients as compared to about 1–2 weeks in HIV-negative patients after initiation of therapy.

One potential weakness of this study is related to the fact that samples could not be obtained at regular intervals during the follow-up. The heterogeneity of samples obtained after different durations in the patients could have affected our analysis. Based on our data, we suggest the use of EIA and PCR, as these are sensitive, specific and valuable aids in establishing diagnosis. These could be of great help in early detection of cryptococcosis, thus reducing morbidity and mortality in the present Indian setting where the incidence of the disease is rising alarmingly.

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