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Research Article

Correlation of Cryptococcal Antigen Assay with C-reactive Protein as Serum and Urine Biomarker in Cryptococcal Meningitis: Experience in a Tertiary Hospital

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Abstract

Aims and Objectives:

The incidence of cryptococcal meningitis caused by *Cryptococcus neoformans* has risen markedly over the past 20 years as a result of the HIV epidemic and increasing use of immunosuppressive therapies. The objectives of this study were to isolate and identify *Cryptococcus neoformans* from clinically suspected cases of fungal meningitis by conventional techniques and evaluate the role of C-reactive protein (CRP) as a serum or urine biomarker for the diagnosis and monitoring of patients with cryptococcal meningitis.

Materials and Methods:

Direct microscopic examination of the CSF samples from clinically suspected cases of fungal meningitis was done by India Ink staining for the capsule demonstration and isolation of the *Cryptococcus neoformans* was done by inoculation of the sample on Sabourauds dextrose agar. Latex agglutination test for the presence of cryptococcal antigen was done on sera, CSF and urine samples. C Reactive Protein levels were estimated in sera and urine.

Result:

Cryptococcal meningitis was diagnosed in 12 cases by culture and/or India Ink staining and/or latex agglutination assay for antigen detection in CSF. Only 8 (66.67%) and 1 (8.33 %) out of 12 samples

were positive for cryptococcal antigen in sera and urine respectively. Whereas all the 12 patients were positive in the sera for CRP above the detection threshold limit, only 1 (8.33 %) patient had raised CRP in urine. CRP was raised two weeks after initiation of antifungal therapy in 3 of the above 12 sera and all these 3 cases turned out to be recurrent cases of cryptococcal meningitis.

Conclusion

Given the high incidence, morbidity and mortality associated with cryptococcal meningitis, it would be ideal if a screening test could be used to exclude this diagnosis based on the presence of biomarkers in serum or urine which would mean less discomfort for the patient in addition to decreased laboratory examination costs.

Keywords:
Cryptococcal Meningitis; Cryptococcus Neoformans; C-Reactive Protein; Cryptococcal Antigen; Biomarkers; Latex Agglutination

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Introduction

Cryptococcosis, caused by Cryptococcus spp., is one of the most common opportunistic infections among human immunodeficiency virus (HIV) infected individuals. [1] *Cryptococcus neoformans* (*C. neoformans*) is a ubiquitous encapsulated yeast that causes significant infections, which range from asymptomatic pulmonary colonization to life threatening meningoencephalitis. The incidence of cryptococcal meningitis caused by *C. neoformans* has risen markedly over the past 20 years as a result of the HIV epidemic and increasing use of immunosuppressive therapies. [2] Although cryptococcal meningitis is generally thought to be associated with immunocompromised patients, the incidence among immunocompetent patients has reportedly risen over recent years. [3] Globally, approximately 1 million new cases of cryptococcal meningitis occur per year, with more than 600 000 deaths. [4] An estimated 88% of global cases and more than 90% of deaths from cryptococcal meningitis occur in sub-Saharan Africa and Southeast Asia. [5]

Although antiretroviral therapy (ART) availability has led to improved cryptococcal meningitis survival, mortality in such cases is still high, in part due to paradoxical HIV immune reconstitution inflammatory syndrome (IRIS), an exaggerated inflammatory response causing a subset of persons with recent cryptococcal meningitis to paradoxically deteriorate on ART in the presence of improving immune function reported as 10%–42% among ART-naïve persons with cryptococcal meningitis. [6], [7] In HIV-infected patients, the disease is associated with profound immunosuppression, usually occurring at CD4 counts <100 cells/µl. There is a greater likelihood of involvement outside the CNS and relapse if antifungal therapy is discontinued prior to effective antiretroviral therapy. Compared with

HIV-negative patients, the presentation tends to be more acute, and is associated with higher serum cryptococcal antigen titres and a poor CSF inflammatory response (white blood cell count <20/µl).

The clinical signs and symptoms of *C. neoformans* meningitis are indistinguishable from those of many other causes of meningitis. Early diagnosis of cryptococcal infection is therefore necessary for appropriate management. The diagnosis of cryptococcal meningitis presently rests on lumbar puncture demonstrating cryptococcal organisms by smear, antigen or culture. This is an invasive and costly procedure and is associated with patient discomfort. Culture, though being considered a gold standard, requires laboratory infrastructure and is not highly sensitive as it requires a sufficient amount of sample which often becomes a limiting factor in meningitis cases especially in children. Diagnosis, though rarely a problem in HIV-associated cryptococcal infection (since the high organism load means that Indian ink preparations of CSF are usually positive, and cryptococcal antigen testing of either CSF or serum has a high sensitivity and specificity), may sometimes be negative and is hard to exclude in non-HIV-associated disease, especially in apparently immunocompetent patients. [8] Large-volume CSF cultures and repeated lumbar punctures may be needed in this setting.

It would be ideal if a screening test could be used to exclude this diagnosis based on the presence of biomarkers on simple blood or urine examination which would mean less discomfort for the patient in addition to decreased laboratory examination costs. The detection of capsular antigen from *C. neoformans* in serum and cerebrospinal fluid (CSF) is a sensitive and specific test for the rapid diagnosis of cryptococcosis and titration of antigen in serum and CSF specimens has been used for diagnosis, prognosis, and monitoring of antifungal therapy. [9] C-reactive protein (CRP) has been established as a marker of an acute phase response or inflammation. Further utility of CRP estimation has been documented in differentiating viral and bacterial meningitis. [10]

The rising incidence of cryptococcosis in India is posing a serious threat. [11] However, the expansion of antiretroviral programmes now raises the prospect of transforming the long-term prognosis of these patients, provided that they survive the acute phase of the illness. Therefore there is an urgent need to define more diagnostic techniques, fungicidal drug regimens, and to improve the understanding of pathogenesis and management of this disease in this setting. With this aim in mind the objectives of this study were to isolate and identify Cryptococcus spp. from clinically suspected cases of fungal meningitis by conventional techniques and evaluate the role of C-reactive protein as serum or urine biomarker for the diagnosis and monitoring of patients with cryptococcal meningitis.

Materials and Methods

A prospective study was done on all the CSF samples received in the Department of Microbiology, UCMS (University College of Medical Sciences) and GTB (Guru Teg Bahadur) Hospital, Delhi; from clinically suspected cases of meningitis during one year period (January 2010 to 2011). Informed consent and Institutional ethical clearance were obtained. Detailed history and other relevant information of all the cases were recorded on a predesigned performa. Twenty five age and sex matched healthy controls were included in the study.

Inclusion criteria: (i) all clinically suspected adult patients of meningitis admitted in the Medicine

Ward/Emergency, GTBH.

Exclusion criteria: (i) patients with severe sepsis/ multi organ failure proved clinically or by biochemical

blood tests. (ii) pediatric patients

Sample collection and processing

Clinical samples:

1. At least 4-5 ml of CSF sample was collected from all the suspected cases of meningitis.

2. 5 ml peripheral venous blood sample was collected from all the cases and controls and sera were separated.

3. 5 ml of urine sample was collected from all the cases and controls.

Laboratory tests:

1. Direct microscopic examination of the CSF samples by India Ink preparation for capsules of *C. neoformans* was done.

2.Isolation of *C. neoformans* by inoculation of CSF samples on SDA (sabouraud dextrose agar) was done. Identification was done by the routine protocol for fungal identification followed in our laboratory. [12]

3. Antigen detection for *C. neoformans* was done by latex agglutination assay (Cryptococcal Ag Latex Agglutination System (CALAS^{R}) Meridian Bioscience, Inc. Cincinnati, Ohio) on all the CSF samples, sera and urine samples.

4. C-reactive protein (CRP) levels were tested in sera and urine samples by latex agglutination kit (RHELAX-CRP, Tulip Diagnostics (P) LTD., Goa India) at the time of recruitment and 2 weeks after starting antifungal therapy.

Results

Out of 1080 CSF samples from suspected cases of meningitis that were received during January 2010 to 2011 in the Microbiology Laboratory, 54 samples were from patients strongly suspected of fungal meningitis. Ten samples (18.5%) were positive for Cryptococcus by India ink staining, whereas 12 (22.2%) were positive on culture and latex agglutination for cryptococcal antigen in CSF. Out of the total of 12 cases of diagnosed cryptococcal meningitis, 9 (75%) were HIV reactive and the remaining 3 were immunocompetent.

When latex agglutination for cryptococcal antigen was done in the sera and urine of these 12 patients of cryptococcal meningitis to see whether antigen detection for Cryptococcus had any diagnostic role in samples other than CSF, it was found that 8 (66.67%) and only 1 (8.33 %) out of 12 samples were positive for cryptococcal antigen in sera and urine respectively. The sample which was positive for cryptococcal antigen in urine also had antigen present in the serum.

As regards the C-reactive protein estimation at the time of recruitment in the above patients; whereas all the 12 (100%) patients were positive in the sera for C-reactive protein above the detection threshold limit, only 1 (8.33 %) patient had C-reactive protein above the threshold limit in the urine. This was the same patient who also tested positive for the cryptococcal antigen. However after 2 weeks of initiation of antifungal treatment, whereas none of the urine samples had detectable levels of C-reactive protein, only 3 (25%) sera were positive for C-reactive protein. These 3 cases were the ones that had a recurrent episode of cryptococcal meningitis. None of the 25 controls had detectable levels of C-reactive protein in either sera or urine samples.

Discussion

The primary pulmonary infection caused by *Cryptococcus neoformans* (*C.neoformans*) is frequently asymptomatic and may be eradicated or contained within granulomata. However, depending on host factors, inoculum, and possibly a number of virulence factors, *Cryptococcus neoformans* may disseminate, especially if specific T-cell immunity is compromised, either acutely or after a period of latency to extrapulmonary sites; with a particular predilection for the brain.

Meningitis is the most frequent manifestation of cryptococcosis. Clinical features are not specific and patients usually present with headache, fever, malaise and altered mental status over several weeks. Signs are often absent, but complications due to raised intracranial pressure are common. Untreated cryptococcal meningitis is uniformly fatal, although survival can range from years in those without apparent immunocompromised to only a few weeks in HIV-associated infection. [13]

In our study, the ages of patients diagnosed with cryptococcal meningitis were very diverse (median 47.5 years; range 25-73 years) and men were dominant. Fever, headache and altered mentation out of many various symptoms were the most common symptoms, which is consistent to the earlier reports. [14]

Detection of cryptococcal antigen by latex agglutination and India Ink staining for cryptococcal capsules were important for diagnosis in our study because 10/12 (83.3%) and 12 (100%) of the CSF samples positive for culture were also positive by India Ink staining and latex agglutination test respectively. Previous studies have reported that the sensitivities, specificities and false positivities of the cryptococcal antigen detection test are in the range of 93-100%, 93-98% and approximately 0-0.4%,

Table 1. Laboratory details of the patients of cryptococcal meningitis diagnosed by India Ink staining and/or culture and/or latex agglutination (n=12).

Case no.	India Ink staining	Culture on SDA	Latex agglutination for cryptococcal antigen			C-reactive protein estimation			
			CSF	Urine	Serum	At recruitment		After 2 weeks of antifungal therapy	
						Serum	Urine	Serum	Urine
1	+	+	+	+	+	+	+	+#	-
2	+	+	+	-	+	+	-	+#	-
3	+	+	+	-	+	+	-	+#	-
4	+	+	+	-	+	+	-	-	-
5	+	+	+	-	+	+	-	-	-
6	+	+	+	-	+	+	-	-	-
7	+	+	+	-	+	+	-	-	-
8	+	+	+	-	+	+	-	-	-
9	+	+	+	-	-	+	-	-	-
10*	+	+	+	-	-	+	-	-	-
11*	-	+	+	-	-	+	-	-	-
12*	-	+	+	-	-	+	-	-	-

* Immunocompetent patients
# Recurrent cases

respectively, and the Indian ink test is positive in 70-90% of the patients with HIV and in only 50% of the patients without HIV. [14], [15], [16] Direct examination by India ink is a rapid but relatively insensitive method. It is positive in at least 50% of cases of cryptococcal meningitis in non-HIV-infected populations and 74 to 88% of cases in HIV-infected patients. In addition, CSF chemistries and cell counts are often within normal parameters, precluding the use of these methods as rapid indicators of infection in HIV-infected individuals. Serum and urine samples, however, have not shown in our study to be very reliable biomarkers for the detection of cryptococcal antigen at the time of initial investigation of such patients as 8/12 (66.67%) and only 1/12 (8.3 %) of cases were positive for cryptococcal antigen in sera and urine respectively. A previous report from Delhi has reported sensitivity, specificity, positive predictive value and negative predictive value of latex agglutination test on serum and urine as 100, 93.3, 94.6, 100 % and 100, 90, 90.3, 100 % respectively. [17]

Early diagnosis of cryptococcal meningitis is advocated, since late presentations are often associated with higher rates of morbidity and mortality. [18] Cryptococcal antigens remain detectable after therapy when both fungal stain and culture turned negative. A decrease in cryptococcal antigen titers can be used to monitor the antifungal therapy efficacy but cannot be used as an index of cure. Hence, cryptococcal antigen latex agglutination test is highly specific to the diagnosis of cryptococcal meningitis; however, its significance for monitoring therapy is limited. Since the goal of therapy for cryptococcal meningitis is to eradicate the pathogenic fungi from the patients' bodies, the therapy protocols should be based on symptoms and physical signs and on examination for the organism from CSF. Although some studies have suggested screening for serum cryptococcal antigens as a method for earlier detection of

cryptococcal disease among HIV patients, this approach has shown mixed results due to the difficulties in obtaining repeated CSF samples, more so because of the lower sensitivities of these antigen detection assays in other samples like sera and urine as also seen in our study. Hence a suitable biomarker is highly desirable whose presence in samples other than CSF could correlate well with the conventional systems of diagnosis for cryptococcal meningitis.

In our study we have found CRP is a highly useful diagnostic marker in serum for patients of cryptococcal meningitis especially at the time of initial investigation. A previous study, however, concluded serum CRP as a poor screening tool in patients with cryptococcal meningitis on the day of presentation and reported a sensitivity of 64.71% for elevated CRP for the diagnosis of cryptococcal meningitis diagnosis. [19] Also in our study the CRP was raised in all the 3 cases that recurred which suggests that serum CRP could serve as a potentially useful biomarker for monitoring the patients of cryptococcal meningitis for any repeated episode. All the 3 cases that recurred were HIV positive, and all our recurrent cases typically manifested approximately one to two months after initiation of HAART as reported by others also. All these three cases that recurred had detectable levels of CRP in their sera 2 weeks after initiation of antifungal therapy. Uncommon presentations due to cryptococcus may be on the horizon since the advent of HAART, which often reconstitutes or maintains cellular immune functions among HIV infected persons. [20] Other atypical manifestations of cryptococcal disease have been reported in association with the introduction of HAART as part of an RIS. In all the 3 cases, except for the culture which was negative, antigen detection test and India ink staining were positive. This is in contrast to some other reports that suggest the serum cryptococcal antigen may be negative in such cases, making the diagnosis challenging. [21] Since cultures were negative for all the three cases that recurred, there might also be a possibility of IRIS as a cause of recurrence in all these cases since diagnosis of such syndromes is often by exclusion and requires the onset of symptoms or signs after initiation of effective antiretroviral treatment, without alternative explanation, attributable to increased reactivity to cryptococcal antigens. The role of immune reconstitution in all these case is supported by the development of an exuberant inflammatory response as documented by the increased levels of CRP, and the temporal relationship of this response to the initiation of HAART. This is further substantiated by a previous report [22] that states that persons who developed IRIS had elevated levels of CRP before ART initiation, and at the time of IRIS events because a failure of antigen clearance promotes generalized inflammatory signaling to then occur, specifically with IL-6 which is a primary stimulant of hepatic CRP production. Given the high incidence, morbidity, and mortality associated with cryptococcal meningitis along with associated IRIS, identifying patients at risk by use of such biomarkers may enable interventions to improve management.

Competing Interests

The authors have declared that no competing interests exist.

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