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Research Article

EVALUATION OF DIFFERENT METHODS FOR RAPID DIAGNOSIS OF CRYPTOCOCCAL MENINGITIS PATIENTS AT TERTIARY CARE HOSPITAL

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A B S T R A C T

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 diagnosis by Latex agglutination test (LAT), cryptococcal antigen (CrAg) lateral flow immunochromatographic assay (LFA), we assessed diagnostic performance of cerebrospinal fluid (CSF) culture, CrAg latex agglutination, India ink microscopy, and CrAg LFA for 30 samples with suspected cryptococcal meningitis during the study period. CrAg LFA had the best performance (sensitivity 100%, Specificity 80%) for CSF, Serum as well as urine samples. India ink Microscopy was 66.7% sensitive. CrAg Lateral Flow Assay (LFA) is a simple, rapid and sensitive test for the early detection of cryptococcal antigen in clinical samples like CSF, Serum and urine and may be considered as an aid in establishing diagnosis when culture is positive/negative. CrAg LFA could be a major advance diagnostic approach in urine samples as non-invasive technique for Cryptococcal meningitis in resource limited setting.

INTRODUCTION

Cryptococcal meningitis (CM) is caused by *Cryptococcus neoformans* which is the most common fungal pathogen of the central nervous system (CNS).[1] It generally develops in immunocompromised hosts and around 15% of death accounts for AIDS-related infection. The majority of cases are found in sub-Saharan Africa[2] Across the worldwide, approximately 100000 new cases are reported for the infectious disease and additionally, around 600000 patients die each year with the same cause[3]. In India and in Southeast Asia have a substantial burden of CM cases, i.e. it accounts for 3% cases per year with high mortality rate of 20%-30%[4,5]. Among the opportunistic infection, it is considered to be one of the leading cause or mortality amongst persons with advanced HIV in the developing world[6,7,8,9]. The diagnosis of CM in HIV infected persons is based upon several modalities. The baseline investigations initiate with the clinical signs and symptoms, microscopy, culture, or antigen detection.

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Most commonly, India ink staining is being carried out for identifying the *Cryptococcus*using the cerebrospinal fluid (CSF) sample, but the sensitivity of India Ink microscopy is only <86%, but can be lower in HIV-negative patients in association with a low fungal burden[10,11,12] In addition to this, culture and sensitivity is considered as gold standard for the diagnosis of CM, but it has certain limitations[13]. Firstly, culture results take long time (7 days to grow) and furthermore, it has to be incubated for upto 10 days for a reliable quantitative count. Secondly, it may also lead to false positivity and negative results due to low fungal and/or previous exposure to antifungal therapy. Low fungal burden is one of the common factors, which hinders the sensitivity of India Ink staining and culture results[14,15] Therefore, direct microscopy and culture are specific but the sensitivity is poor (50-80%) [16].

Serology, a rapid means of diagnosis, is an indirect and adjunct or complementary procedure to support clinical diagnosis, especially when patient is on treatment.[14,15] Latex agglutination test (LAT) is the most commonly used serological method due to its simplicity in performance[17,18] Detection of cryptococcal capsular antigen has sensitive and specific of 83% to 97% and

the specificity from 93% to 100% [19]. However, it has certain limitations of false positivity[20,21] unacceptably high rates of false negativity[22,23.] and the difficulty of its interpretation in borderline cases. Enzyme immunoassay (EIA) is another serological tool for detection of capsular polysaccharide antigens of *C. neoformans* in CSF. This is a rapid test that provides visual and numeric result in less than an hour without pre-treatment of the specimen[24,25] There are limitations of serological tests due to the possibility of false positivity with few other microbes and some other disease conditions.

Coupled with the advantages of serological tests, the rapid microscopic tests for cryptococcal meningitis are negative in few patients, and the diagnosis is delayed for 24-72 hours while awaiting a confirmation by culture of cryptococci from CSF.

In order to overcome the shortcomings, related to the diagnosis of CM, nowadays Cryptococcal Antigen Lateral Flow Assay (CrAg LFA) is being used. Recently developed, lateral flow immunochromatographic detection test, detects the cryptococcal antigen. The advantageous points for this test is that it is quite economical, rapid (≤ 10 minutes) and can be performed without electricity[26]. In addition to these, it provides both qualitative and semi-quantitative results. In cases of CSF samples Cr-Ag Latex reported to high sensitivity and specificity of 93-100% and 93-98%[27] CrAg-latex has reduced sensitivity for CrAg of serotype C (i.e. *C. gattii*). The principle behind this technique is that it uses monoclonal antibodies which facilitates for uniform reagent quality and performance. It uses a combination of two monoclonals. First monoclonal antibody is highly reactive with CrAg of serotypes A,B and C and the second monoclonal antibody is highly reactive with CrAg of serotypes A and D. Therefore, when the test is performed and the sample is positive for Cryptococcal antigen suspended, gold-conjugated antibodies bind to the antigen. The gold-antibody-CrAg complex migrates by capillary action up the test trip, interacts with immobilized monoclonal antibodies against CrAg, and forms a red line.

MATERIAL & METHODS

The retrospective study was conducted in the Dept. of Microbiology of Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow. The study was ethically approved by the Institutional Committee (IEC 6/14) and the study was carried out from July 2015 to July 2018. The study set comprised of 71 subjects, which was categorised into two groups, CM cases (n= 31; 43.7%) and the control group (n= 40; 56.3%). The subjected were included in the study were, as per the inclusion and exclusion. Inclusion criteria were based on the case definition which was rely upon clinical, laboratory and/or radiological criterion. Clinical picture compatible with a diagnosis of cryptococcal meningitis (any evidence of fever/ headache/meningismus/altered mental status or any other neurological manifestation) with either a cerebrospinal fluid (CSF) abnormal biochemistry and/or pleocytosisand with a cryptococcal antigen titer of ≥ 1:8 and/or Positive India Ink preparation for capsulated yeast cells and/or a Positive CSF culture yielding *Cryptococcus neoformans* [28.] On the other hand, control group was comprised of patients suffering from migraine, microcytic anaemia, lumbar arachnoiditis, cranial nerve palsy, paraparesis and hemiparesis.

Sample Collection and Processing

All the surplus CSF samples collected for Microbiological & Pathological evaluation from the Neurology Department of Dr.RMLIMS/ KGMU for diagnostic, therapeutic & prognostic purposes of suspected cases of sub-acute meningitis & chronic meningitis patients as well as from diseased controls, in the Microbiology & Pathology Department of Dr. Ram Manohar Lohia Institute of Medical Sciences. CSF (2-3 ml) was collected under standard protocol from patients and also from control population under aseptic conditions by lumbar puncture involving minimum or no risk.CSF samples of disease control group were collected from a cluster of individuals who were suspected to be suffering from neurological disorders other than meningitis. The informed consent of the patients was obtained, after which standard history-taking examination and baseline investigations (including radiological investigations wherever required) were carried out.

The CSF samples were centrifuged and deposit was processed for fungal culture, negative staining or India Ink Preparation (encapsulated, spherical, budding yeast seen), Gram's staining and culture. The deposit of CSF was inoculated in two sets of Sabouraud's Dextrose agar (SDA), one incubated at 25^0C and other at 37^0C. Sample is also inoculated on Blood Agar and Brain heart Infusion Broth (BHI) to confirm the bacterial growth. Fungal cultures were observed for growth for 4 weeks, to observe the positivity of the cultures, which appears in mucoid and creamy whitish Coloured colonies. Supernatant of the CSF sample was used for Latex Agglutination Test (LAT). LAT assays were performed with Cryptococcal Antigen Latex Agglutination System (CALAS) using (Bio- Rad, Pastorex^{TM}Crypto plus). The test was performed according to guidelines of the manual. Further, the LAT was also performed in the CSF, serum and urine samples, using theCryptococcus antigen (CrAg) Lateral Flow Assay (LFA), The test was kit based and was carried out using BIOSYNEX®CryptoPS (BIOSYNEX SA). It is an immunochromatographic dipstick assay that also detects antigen with qualitative and semiquantitative results. The clinical signs and symptoms, taken into consideration were statistically analysed using SPSS (Statistical Package for the Social Sciences) version 16.0 to differentiate the case and control group and to observe the clinical relevance.

RESULT

On the basis of the statistical analyses, majority of the cases were found to be in the age group of 31-45 years i.e. (n=13; 56.5%), which was found to be significant (*p value*= 0.02) (Supplementary Table S1). Gender wise distribution of cases results to maximum number of male patients (n=24; 48%), though there was no significance was observed (Supplementary Table S2). Among the clinical signs and symptoms, fever and headache were significantly marked for the differentiation of cases and control groups (Supplementary Table S3). Cryptococcal meningitis is a frequent HIV-related opportunistic infection, hence, in our study, it depicted, as 11 subjects from the cases and 2 subjects in the control group were HIV positive. The baseline investigations initiate with the preparation of wet mount and India ink, which results to be sensitive in 23 (95.8%) and 25 (80.6%) (Supplementary Table S4) cases respectively which was highly significant (*p value*= 0.001).

Supplementary Table S1: Age wise distribution of Cases and Control group

Age	Group				
	Cases(n=31)		Controls(n=40)		
	No.	%	No.	%	p-value
18 to 30 yr	5	25	15	75	
31 to 45 yr	13	56.5	10	43.5	
46 to 56 yr	6	85.7	1	14.3	
57 to 67 yr	6	40	9	60	0.024
>67 yr	1	16.7	5	83.3	

Supplementary Table S2 Gender wise distribution of Cases and Control group

Gender	Group			
	Cases(n=31)		Controls(n=40)	
	No.	%	No.	%
Male	24	48	26	52
Female	7	33.3	14	66.7

Supplementary Table S3 Clinical Signs and symptoms among Cases and Control group

Characteristic	Cases (n=31)		Controls (n=40)	
	No.	%	No.	%
Fever	29	93.5	27	67.5
Headache	25	80.6	17	42.5
Convulsions-generalised	1	3.2	4	10.0
Convulsions-focal	2	6.5	2	5.0
Nausea/vomiting	20	64.5	24	60.0
Altered sensorium	14	45.2	10	25.0
Abnormal movement	3	9.7	3	7.5
Signs of meningeal irritation	0	0	3	7.5
Evidence of source of infection	1	35.5	38	95
Sensitivity of light	1	3.2	0	0
Stuff neck	0	0	3	7.5

p-value is significant to fever and headache.

Supplementary Table S4 Positive results of HIV cases, Wet Mount and India Ink

Characteristic	Group				
	Cases(n=31)		Controls(n=39)		
	No.	%	No.	%	p-value
1. HIV					
Positive	11	84.6	2	15.4	0.001
Negative	20	34.5	38	65.5	
2. Wet mount					
Yes	23	95.8	1	4.2	<0.001
No	8	17.4	38	82.6	
3. India Ink					
Positive	25	80.6	0	35.7	<0.001
Negative	6	19.4	39	100	

Further, followed with culture and sensitivity, out of 31 cases, 26 (83.9%) culture results were found to be positive with organism identified from the isolates was *Cryptococcus neoformans*. CrAg LAF was conducted in CSF, serum and urine samples of all the subjects of cases group. Therefore, 10 (33.3%),12 (40.0%) ,22 (100%) were positive and 12(40.0%), 10(33.3%) and nonewere strongly positive in CSF, serum and urine samples respectively while remaining samples resulted as negative for the test (Supplementary Table S5). The sensitivity and specificity obtained from CrAg LFA 100% and 80% in CSF samples. In contrast to CrAg LFA, CALAS was also performed in the CSF samples of all the cases as well as control subjects, 30 cases proved to be positive in the cases group while in control all 40 subjects deciphered with negative results. Similarly, sensitivity and specificity was calculated and depicted as 100% and 80%. Majority of cases i.e. 22 subjects (73.3%) were resulted as 1:1000 in CALAS titre, which deciphered as high fungal burden. On the basis of biochemical parameters, CSF cell count, CSF protein and CSF sugar were analysed, among them CSF cell count and CSF sugar were marked significantly (Supplementary Table S6).

Supplementary Table S5: Culture Sensitivity among Cases and Control group

	Group				
	Cases(n=31)		Controls(n=40)		p-value
CSF culture for fungus after incubation for 15 days at 37^0C					
Positive	26	83.9	0	0	
Negative	5	16.1	40	100	<0.001

Supplementary Table S6: Sensitivity and Specificity of biofluids against CrAg LFA

	India Ink (n=30)						
1. Crypto PS kit for CSF	Positive		Negative		Total		
	No.	%	No.	%	No.	%	p-value
Positive	20	100	2	20	22	73.3	
Negative	0	0	8	80	8	26.7	<0.001
2. Crypto PS kit for Serum							
Negative	0	0	8	80	8	26.7	
Positive	10	50	2	20	12	40	<0.001
Strong Positive	10	50	0	0	10	33.3	
3. Crypto PS kit for Urine							<0.001
Negative	0	0	8	80	8	26.7	
Positive	20	100	2	20	22	73.3	

Sensitivity and Specificity for all body fluids (CSF, Serum and Urine) were 100% as well as 80%.

Characteristics	Group				
	Cases (n=31)		Controls (n=38)		
1. CSF cell count	No.	%	No.	%	p-value
0-5/cm	7	22.6	18	47.4	
6-<50/cm	8	25.8	12	31.6	0.029
50-<100/cm	10	32.3	2	5.3	
100-<500/cm	4	12.9	5	13.2	
>500/cm	2	6.5	1	2.6	
2. CSF Sugar					
10-30mg/dl	10	32.3	3	7.9	
31-50mg/dl	13	41.9	9	23.7	
51-70mg/dl	4	12.9	16	42.1	0.005
71-90mg/dl	1	3.2	6	15.8	
91-120mg/dl	3	9.7	4	10.5	

DISCUSSION

There are various methods present for the diagnosis of cryptococcal meningitis but, CrAg LFA proved to be one of the rapid and accurate methods. Usually, in most of the studies CSF and serum samples has been used for the diagnostic purpose[26, 29]. Recently, in few of the studies, other biofluids like serum and urine are also taken into consideration to CSF. The CrAg LFA test is based on the principle of immunochromatographic assay impregnated with monoclonal antibodies with the ability to detect the capsular polysaccharide antigen of *Cryptococcus neoformans* and *Cryptococcus gattii*[30]. In a previous study, it was reported that CrAg in serum was found to be positive in all subjects, while CrAg positivity in CSF of some patients. It was also reported that urine LFA can have false positives, thus any positive result should always be confirmed in serum[31] . In one of the earlier studies, it was summarized that positive urine titres and cultures, may be indicative and sensitive for cryptococcal antigen in urine samples[32,33].Similarly, in our study it was observed that CSF (n=22; 100%) and urine (n=22;100%) samples both gave positive against the same patients and in cases of urine samples, all the subjects were found to be with high titres Among urine samples: n=22;100% were positive and none was strong positive; while in CSF sample: n=10;33.3% were positive and n=12;40% were strong positive. Our study also proved high sensitivity (100%) and specificity (80%) in urine samples. Considering urine samples, for screening of Cryptococcal meningitis could advantageous in various ways, as easier sample collection, non-invasive procedure, could be possible in limited resource settings[34]. Our study also demonstrates the potential value of testing urine samples against CrAg. Therefore, this diagnostic strategy may help to screen out patients presenting with symptoms of CM in preliminary stage or rural clinics where lumbar puncture may also be avoided.

CONCLUSION

Based on our data, we suggest the use of CrAg Lateral Flow Assay (LFA), as these are sensitive, specific and valuable aids in establishing diagnosis. These could be improvised for early detection of Cryptococcosis, thus reducing morbidity and mortality in the present Indian setting where the incidence of the disease is rising alarmingly. So, in order to overcome the delayed and inaccurate diagnosis, the CrAg LFA test in urine samples could be a promising and reliable diagnostic method against Cryptococcal meningitis. Therefore, Urine screening would be ideal as a non-invasive approach.

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