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Laboratory Diagnosis of Cryptococcal Meningitisin Human Immunodeficiency Virus Infection and Acquired Immune Deficiency Syndrome Patients at Moi Teaching and Referral Hospital

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Abstract:
Cryptococcus meningitis is aserious fungal infectionin HIV patients. India ink is the most used method for rapid detection of Cryptococcus neoformansin CSF specimens. Use of CrAg for laboratory diagnosis of cryptococcus meningitis at M.T.R.H will help reduce mortality. It isimportant therefore to compare the sensitivity of India ink test and CrAgagainst fungal culture for Cryptococcus neoformans in HIV AIDS patients at M.T.R.H. The main objective of the study was to compare the sensitivity of India ink test and CrAg against fungal culture for Cryptococcus neoformans in HIV AIDs patients. The study revealed that CrAg had high sensitivity of 94% and high specificity of 100% compared to 44% sensitivity and 98% specificity of India ink. Level of patients missed for cryptococcus meningitis using India ink was found to be 55.5% compared to 5.6% patients missed by CrAg. Risk ratio of CrAg compared to India ink in this study was 2.12. At 95% confidence interval, the 2.12 estimate is statistically significant at confidence limits of 1.65 and 2.74. In the final analysis, it was found out that cryptococcus meningitis in this study accounted for 18% of defining illnesses in HIV/AIDS patients at M.T.R.H. patients missed for cryptococcosis in the study was higher (55.5%) in India ink compared to that of CrAg. Due to high sensitivity in this study, CrAg test was recommended that it should replace India ink test that was used routinely for diagnosis of cryptococcus meningitis in HIV/AIDS at M.T.R.H.

Keywords: *CrAg, India Ink, HIV / AIDs, Risk ratio, Cryptococcus meningitis*

1. Introduction

Cryptococcosis a life-threatening fungal infectionin HIV patients, (Ellen. Jo Baron, *et al.*,1990).Cryptococcosis is a central nervous system (CNS) fungal infection caused by the yeast referred to as *Cryptococcus neoformans*. This pathogen is ever-present organism acquired by inhalation and also common in soils enriched with bird droppings, fruit skins or juices and unpasteurized milk. Cryptococcus infection begins by inhalation of the fungus into the lungs followed by hematogenous spread to the brain and meninges, (Patrick, *et al.*, 1995).

Cryptococcus neoformans enters body through the lungs, the CNS is the main site of infection in people who are immune competent or immune suppressed, (Mitchell. T. G, *et al.,* 1995). In pulmonary infection, cryptococci spreadbroadly and may cause infection in severalorgans like CNS, prostate, eye, bone and skin. Appearance may be sluggish and non-specific, with symptoms like fever, headache, nausea, vomiting, firmness in the neck, photophobia, cough and distorted mental status, (Woldemanuel.Y, *et al* 2001). The fungus may cause an asymptomatic pulmonary infection followed by the advancement of meningitis in these patients, (Khanna.N, *et al* 1996).

Routine tests done for CSF like cell count, glucose and protein levels are of slighthelp in diagnosing cryptococcal meningitis. CSF of patients with cryptococcus meningitis tends to have low white blood cell counts whereas glucose and protein levels are non-specific. Serum cryptococcal antigen (CrAg) test is used for disseminated cryptococcal because of its high sensitivity whereas CSF CrAg is performed as a confirmatory test for neurological infection. Definitive diagnosis of cryptococcus meningitis in the laboratoryis made by fungal culture (sabouraud dextrose agar) of the CSF at 25^{o}C for 72 hours.

2. Epidemiology

Cryptococcosis is globally distributed caused by yeastcalled *Cryptococcus neoformans.* There of two varieties mainly *C. neoformans var. neoformans* and *C. neoformans var. gattii*and serotypes of *C. neoformans* designated A, B, C, and D. serotypes are based on antigenic determinants on the polysaccharide capsule that cause cryptococcosis. During epidemic, approximately 5-8% AIDS patients develop cryptococcal infection but where antiretroviral treatment (ART) is available, the incidence of cryptococcosis beside other opportunistic infections has decreased,(Hajjeh RA, *et al.,* 1999).

Cryptococcosis has been widelybeen described in patients from Europe and North America and recognized as a prominent AIDS associated opportunistic infection in Sub-Saharan Africa for 10 years. Studies conducted in United States suggest that between 6% and 10% AIDS patients will develop cryptococcus meningitis, (Robert S. Heyderman, et al., 1998).Incidence of cryptococcal meningitis varies from place to place. In 1994 annual prevalence of cryptococcosis was considered to be between 6.1% and 8.5% among HIV-infected people in New York City. In 2006, researchers have estimated about one million infections and half a million deaths from HIV-related cryptococcal meningitis globally. Findings published in the AIDS Journal of the 20th February edition shows that sub-Saharan Africa has the highest cryprococcus meningitis global burden.In Thailand, cryptococcosis accounted for 19% of AIDS-defining illnesses between 1994 and 1998, (Casadevall A., 1994).

Cryptococcal meningitis is now the leading cause of community-acquired meningitis accounting for 20–45% of laboratory-confirmed cases of meningitis in Southern Africa which is ahead of tuberculosis and bacterial meningitis, (Aberg, JA., 2000). In Durban, South Africa, cryptococcal meningitis was the initial AIDS-defining illness in 84% of patients, (Moosa, MY., 1997).

A study reviewing the laboratory detection rates of *Cryptococcus neoformans* using India ink at Kenyatta National Hospital from 1987 to 1997 found a detection rate of 5.2% (76/1462), (FA. Odhiambo, *et al*., 1997). Cryptococcus meningitis was the reason for admission in 12% of the 530 AMPATH- enrolled patients admitted at M.T.R.H medical wards, (Ann. Mwangi, et al., 2008). Therefore, this study seeks to assess the laboratory misdiagnosis of cryptococcus meningitis with specific emphasis on establishing the sensitivity of CrAg and India ink tests used for diagnosis of cryptococcus meningitis in HIV/AIDS patients at M.T.R.H.

2.1. Cryptococcus Meningitis Diagnosis

Clinical presentation of cryptococcal meningitis can be fever, malaise, mild headache, nausea and vomiting with neck stiffness being an infrequent sign. Severe cases can be encephalopathic features like change and confusion which carry a worsened prognosis, (Saag MS., 1992). Dissemination of infection is common in AIDS patients liver and lymph nodes with skin lesions resembling that of *Molluscum contagiosum.* Preface diagnosis of cryptococcal infection is made by identification of the yeast in a compatible clinical setting whereas definitive diagnosis is confirmed by the cultureof specimens, often the cerebrospinal fluid (CSF) or blood.

Laboratory diagnosis of cryptococcus meningitis involves smears, culture, histopathology and serological tests. Direct and indirect techniques for detection of metabolites or DNA can add to diagnostic process. In the case of cryptococcus meningitis, white cell count in CSF is raised with a high number of lymphocytes in non-HIV-associated infection, (Tihana, Bicanic. *et al*., 2004).

Diagnosis is rarely difficult in HIV-associated cryptococcal infection because of the high organism load. India ink preparations of CSF are usually positive and CrAg of either CSF or serum with high sensitivity and specificity. On the other hand, in non-HIVpatients the cultures and antigen tests of CSF may sometimes be negative and the diagnosis is hard to exclude, (Berlin, L. *et al.*, 1989).

For laboratory diagnosis of cryptococcus meningitis, blood or spinal fluid, one of the most accurate and sensitive tests involves looking for cryptococcus antigen. Thus, current test of choice for the laboratory diagnosis of cryptococcal meningitis however is the cryptococcal antigen test, (Murray, PR. *et al.*, 1999). This is because the test detects the presence of the cryptococcal capsule in both CSF and serum specimens and is present in different assay formats.

The principle of the cryptococcal antigen latex agglutination test is based on latex particles which agglutinate with specimens (serum or CSF) containing the appropriate cryptococcal capsular antigens. It's found that the clinical manifestations, CSF routine results and biochemistry examinations for cryptococcal meningitis are similar to those of tuberculous meningitis, viral meningitis and atypical purulent meningitis. Therefore, the diagnosis of cryptococcal meningitis depends on CSF India ink smear, fungal cultures and the cryptococcal antigen latex agglutination test, (**Cunha, B. A.,** 2001). In a study done in South Africa stated CrAg tests can be used in screening patients suspected to have cryptococcus meningitis to help in prevention and giving pre-assumptive treatment would be an attractive strategy, (Joseph, N. *et al.*, 2009).

Culture is performed by streaking medical specimens on SDA. The cultures are incubated both at 25°C and 37°C for colonies appearance within 48hours but may take longer depending on the fungal load. Delayed growth is observed if chloramphenicol is incorporated in SDA media. The colonies appear as soft and creamy white to yellow-brown in texture if considerable capsular material is present (WHO guidelines).Based on the morphology on India ink and gram staining, these colonies show gram-positive spherical budding yeast cells and provisionally identified as encapsulated yeast. The colonies are further confirmed using standard biochemical tests.*C. neoformans* is distinguished from other yeasts by its ability to assimilate or hydrolyze urea to ammonia. *Cryptococcus* has ability to invade the central nervous system (CNS) because of its capacity to synthesize melanin from catecholamines present in this tissue in large concentrations, (Levitz SM., 1991). Melanin production allows birdseed agar to be used as a valuable laboratory screening tool, (Torres-Guererro H. *et al.*, 1994).

In India ink mounts, *C. neoformans* appears round budding yeasts surrounded by a prominent capsule. These yeasts are urease positive, nitrate negative, they don't ferment any sugars and produce achocolate-brown pigment on birdseed agar, (Mitchell, TG. *et al.*, 1995).

Routine screening for Cryptococcus meningitis in asymptomatic patients is never recommended, if a positive titer instead, therapy with fluconazole be initiated, (Feldmesser, M. *et al.*,1996). Although serum CrAg is 99% sensitive, positive result doesn't indicate that CNS invasion is present, on the contrary, a negative serum CrAg result suggests that the patient is unlikely to have CNS disease and may be useful in symptomatic patients screening. Therefore, usefulness of serum cryptococcal antigen in asymptomatic HIV patients as a screening tool has not been studied adequately.

The recent test of choice for the laboratory diagnosis of cryptococcal meningitis is CrAg which detects the presence of the cryptococcal capsule in both CSF and serum specimens and is available in different assay formats, (Holtzer, CD., *et al*. 1998). The

sensitivity of the CrAg is above 90% (99% in some studies done in America) and varies with the presence of poorly encapsulated strains, infection stage, number of organisms present and assay methodology, (Reiss, E.*et al.,* 2002). Although serum is useful for the diagnosis of nonmeningealcryptococcosis, the sensitivity is lower because additional reliable results are obtained with the cryptococcal antigen test, (Warren, NG. *et al*., 2002).

Tannel *et al*., (1994) in North Carolina did a comparative study on concert of commercially available assays on sensitivities and specificities of five kits mainly Crypto-LA,CALAS, Myco-Immune, Immuno mycologics and an enzyme immunoassaykit for detecting cryptococcal antigen. It was found that forfour kits tested for CSF specimens, the sensitivities and specificities were comparable (sensitivity, 93 to 100%; specificity, 93 to 98%) while the sensitivity and specificity of CALAS used for CrAg was 100% and 98% respectively. In Zambia patients with cryptococcal meningitis at University Teaching Hospital in Lusaka reported CSF CrAg and fungal culture has sensitivity less than 95% while India ink had sensitivity of 80%, (Amita Gupta *et al*., 2009).

3. Methodology

This study was carried out at Moi Teaching and Referral Hospital in Eldoret with population of all HIV/AIDS patients admitted in medical wards atMoi Teaching and Referral Hospital with signs and symptoms indicative of Cryptococcus meningitis.

- Cross sectional study and purposive sampling was used.

3.1. Inclusion Criteria.

HIV AIDS patients who were on their first episode of Cryptococcus meningitis disease.
Patients who had written informed consent and signed.

3.2. Exclusion Criteria

HIV/AIDs patients on antifungal treatment for any other fungal disease.
HIV AIDS patients who were on the second episode of Cryptococcus meningitis.
Data was obtained by conducting laboratory procedures with subsequent observations. The following implementation procedures were carried out: -

4. Results

In this study, 100 CSF specimens were obtained from HIV/AIDs patients who were admitted in M.T.R.H medical wards. The samples were subjected to three tests mainly CrAg, culture and India ink. This was done for a period of eleven months (June 2009 to October 2010). In the study, 61% (61/100) of the respondents were males and 39% (39/100) were females. The mean age for both male respondents and female respondents was 44 years.

4.1. Comparison of Frequency of Cryptococcus Meningitis Diagnosis by Crag and India Ink on CSF Samples

From the 100 (61 males and 39 females HIV/AIDs patients suspected and tested for cryptococcus meningitis, a total of 17 patients tested positive for Cryptococcus meningitis by CrAg (Table 2). This was a proportion of 17% confirmation by this test. Among the positives were 7 (7%) females and 10 (10%) were females and 83 (51 males and 32 females) negatives.

India ink yielded 10 positives and 90 negatives. The 10 positives had all tested positive on CrAg (0 female and 10 males). (Table 3)

On culture, 8 positive CSF samples were not in agreement between CrAg and India ink. All 10 CSF samples that were positive for India ink were in agreement with CrAg and culture result while the 17 CSF samples that were positive for CrAg were in agreement with culture results. (Table 4)

Status of diagnosis by CrAg	Male.	Female.	Total.
Positive.	10	7	17
N (%)	(10)	(7)	(17)
Negative.	51	32	83
N (%)	(51)	(32)	(83)
Total tested	61	39	100
N (%)	(61)	(39)	(100)

Table 1: Proportion of Cryptococcus meningitis by CrAg using HIV/AIDs patients

TEST	POSITIVE	NEGATIVE	TOTAL TESTED
CRAG	17	83	100
INDIA INK	10	90	100

Table 2: Comparison between results of CrAg and by India ink on CSF samples

TEST	POSITIVE	NEGATIVE	TOTAL TESTED
CULTURE	18	82	100
CRAG	17	83	100
INDIA INK	10	90	100

Table 3: Comparison between results of CrAg, India ink and culture on CSF samples

→ Determination of sensitivity and specificity of CrAg and India ink in diagnosing Cryptococcus meningitis in HIV/AIDs patients.

		GOLD TEST (CULTURE).		
		POSITIVE.	**NEGATIVE.**	**TOTAL.**
INDIA INK.	**POSITIVE.**	TP = 8	FP = 2	10
	NEGATIVE.	FN = 10	TN = 80	90
	TOTAL	18	82	100
CRAG.	**POSITIVE.**	TP = 17	FP = 0	17
	NEGATIVE.	FN = 1	TN = 82	83
	TOTAL.	18	82	100

Table 4: Sensitivity and specificity of India ink and CrAg against culture

5. Discussion

5.1. Comparison of Cryptococcus Meningitis Diagnosis by CrAg and India ink on CSF Samples

In this study, cryptococcus meningitis was found to be common in M.T.R.H occurring in 18% of HIV/AIDS patients presenting with clinical symptoms for Cryptococcus meningitis. The high rate is not surprising, although the magnitude of the problem had not been clear defined. Most reports on the severity of cryptococcus meningitis in Africa continent have originated from South Africa which accounts for 45% confirmed cryptococcosis cases in the laboratory, (Moosa, MY., 1997), leading to some question ifcryptococcosis is less common in the East and West Africa (Maher. D., 1994). This study results suggest cryptococcus meningitis burden is also enormous in this region similar to that seen in Zimbabwe, (Robert, S. Heyderman., 1998). The incidences of cryptococcal meningitis vary from place to place. In a study done in New York, *C. neoformans* was the most common opportunistic pathogen isolated from CSF samples of HIV infected patients with an incidence of 34.8% (31/89), (Manoharan. G, *et al*., 2001).

In this study, males predominated with 61% out of which 42% were between 36 years and 49 years. This was in consistent with studies done in South Africa, Zambia, Uganda and United States where males aged between 35 years and 49 years predominated in the studies. Even though more men are reported to develop cryptococcal disease, the male-to-female ratio essentially is 1:1, when adjusted for the male predominance in HIV infection. From this study, 10 males were detected to be positive for India ink while no female's patient detected positive for Indian ink. Little known why males are more prone to *Cryptococcus neoformans* fungi unlike in females. Cryptococcosis in children with AIDS is less common with a prevalence of about 1.4%, (Abadi J., 1999).

This study CrAg results were compared with those of India ink. The results showed a wide disparity, out of 10 CSF samples that tested positive on India ink, only 8 confirmed positive on CrAg. This was further confirmed positive for cryptococcus meningitis on fungal culture. All 17 patients who tested positive for cryptococcus meningitis on CrAg, tested positive on fungal culture (gold test). This indicated that CrAg detected more HIV/AIDS patients who had cryptococcus meningitis disease compared to India ink.

From this study, India ink had the ability to detect 80 true negatives while CrAg test had the ability to detect 82 HIV/AIDS patients who did not have cryptococcus meningitis which was in agreement with negative patients tested on fungal culture. True negatives indicated healthy people correctly identified as healthy while false negative indicated sick people incorrectly identified as healthy. From the above findings, the level of patients who were missed for cryptococcus meningitis by India ink was 55.5% (10/18) compared to 5.6% (1/18) missed by CrAg.

Based on this study, it was suspected that more missed cases were to be reported at M.T.R.H if continued use of India ink for diagnosis of cryptococcus meningitis. According to Murray and co-workers (1999), India ink was less accurate than CrAg because of its low sensitivity. Therefore, these results showed that the sole reliance on India ink as a test for cryptococcus meningitis at M.T.R.H was unreliable.

True positive indicated that the sick people were correctly diagnosed as sick by the tests. In this study, out of 100 CSF samples diagnosed for cryptococcus meningitis, CrAg test had 17 true positive while India ink gave 8 true positives. The detection rate of *Cryptococcus neoformans* using India ink test in this study was 8% (8/100) slightly higher than that of a study done at Kenyatta National Hospital from 1987 to 1997 that was 5.2% (76/1462), (FA. Odhiambo *et al*., 1997).

It was found that India ink had two false positives indicating that out of 100 HIV/AIDS patients who were clinically suspected to have cryptococcus meningitis,2.44% (2/82) were incorrectly identified to have the disease by India ink test unlike CrAg that did not identify any false positive. In addition, out of 1734 HIV/AIDS patients clinically diagnosed for cryptococcus meningitis in year 2007, 35 patients were likely to be incorrectly detected by India ink to have the disease. Patients whose India ink results were false positive, no confirmatory test on Crag was requested meaning that some clinicians rely on routine test and the patients were put on medication wrongly. Based on this data obtained and analyzed, it was evident that laboratory diagnosis of cryptococcus meningitis by India ink had considerable financial implication to the patients.

5.2. Determination of Sensitivity and Specificity of CrAg and India ink in Diagnosing Cryptococcus Meningitis in HIV/AIDs Patients

The main objective of this study was to compare the sensitivity of CrAg and India ink. Results of this study showed that India ink had a lower sensitivity of 44% compared to that of CrAg that was 94%. The specificity of India ink was 98% which was slightly lower than 100% specificity of CrAg. This indicated that CrAg had a greater chance of identifying those patients who had cryptococcus

meningitis and had a greater ability of identifying patients who did not have the disease compared to India ink. This was in conscience with studies from North Carolina (Tannel, et al., 1994) and South Africa (Joseph, N. *et al*., 2009) where the sensitivity and specificity of CALAS (CrAg kit) was 100%; 98% (N. Carolina) and 100%; 96% (S. Africa) respectively, whereas the Zambian study conducted in 2001 by P. Mwamba and his co-workers gave sensitivity on CrAg as more than 95% and 60% sensitivity for India ink.
In this study, CALAS assay was used for CrAg test which gave sensitivity of 94% and specificity of 100%. In a study done by Tannel et al., (1994) for different CrAg assays or five latex agglutination test kits (Calas, Crypto-LA, Myco-Immune, Immy and an enzyme immunoassaykit) which gave a range of sensitivities between 93% to 100% and specificities between 93% to 98% whereas CALAS assay giving sensitivity of 100% and specificity of 98%. The variations were attributed by the presence of poorly encapsulated strains, infection stage, number of organisms present and the assay methodology, (Reiss. E, *et al*., 2002).
Sensitivity of CrAg in this study was compared to that of India ink using culture as the gold standard which had sensitivity of 95% in a study done in Zambia at University Teaching Hospital by Amita Gupta and his co-workers in 2009. Definitive diagnosis is confirmed by the culture of cerebrospinal fluid (CSF) and preliminary diagnosis of cryptococcal infection is made by identification of the yeast in a compatible clinical setting (Chuck SL. *et al*., 1989).
The risk ratio of CrAg compared to India ink in this study was 2.12 meaning that CrAg screening test is twice as good as the India ink test (or CrAg is two times better than the India ink) in detecting the disease in the subjects who were actually suffering from the Cryptococcus meningitis. This estimate is statistically significant at 5% level of significance.

6. Conclusion

CrAg test had high sensitivity (94%) and high specificity (100%) compared to India ink which had specificity of 98% and sensitivity of 44%.
CrAg test was found to be twice as good as the India ink test in detecting Cryptococcus meningitis in HIV/AIDS patients.
The prevalence of Cryptococcus meningitis at M.T.R.H was found to be at 18%.
Based on laboratory findings in this study, the level of laboratory misdiagnosis (false negatives) of cryptococcosis at M.T.R.H was found to be 55.5% while the level false positive was found to be at 2.44% implying that India ink was not a reliable test for diagnosis of cryptococcus meningitis in M.T.R.H.

7. ABBREVIATIONS

- HIV –- Human Immuno deficiency Virus
- AIDS - Acquired Immune Deficiency Syndrome
- CrAg - Cryptococcus Antigen.
- M.T.R.H - Moi Teaching and Referral Hospital.
- ARV – Antiretroviral.
- CSF – Cerebrospinal Fluid.
- CNS – Central Nervous System
- IREC – Institutional Research and Ethics Committee.
- CALAS – Cryptococcus Antigen Latex Agglutination System.
- DNA – Deoxy-Nucleic Acid.
- WHO – World Health Organization.
- SDA – Sabouraud Dextrose Agar.

8. Definition of Terms

Cryptococcus neoformans is an encapsulated yeast that can live in both plantsand animals. Cryptococcosis, or cryptococcal disease, is a potentially deadly fungal disease. It is as a result of inhaling the fungus Cryptococcus neoformans. It is one of the diseases most frequently affecting AIDS patients. It may be limited to the lungs, but frequently spreads throughout the body. Immuno-competence is the ability of the body to produce a normal immune response following exposure to an antigen. Immuno-competence is the opposite of immunodeficiency / immuno-incompetent / immuno-compromised. Immunodeficiency (or immune deficiency) is a state in which the immune system's ability to fight infectious diseases is compromised or entirely absent.
India ink (or Indian ink in British English) is a simple black ink used for writing and printing anddrawing, especially when inking comic books and comic strips. Sensitivity (also called recall rate in some fields) measures the proportion of actual positives which are correctly identified or it is the proportion of persons with disease who are correctly identified by a screening test or case definition as having disease.
Specificity measures the proportion of negatives which are correctly identified or it is the proportion of persons without disease who are correctly identified by a screening test or case definition as not having disease.

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