SENSITIVITY PATTERNS, SEROTYPES OF CRYPTOCOCCUS NEOFORMANS AND DIAGNOSTIC VALUE OF INDIA INK IN PATIENTS WITH CRYPTOCOCCAL MENINGITIS AT KENYATTA NATIONAL HOSPITAL.

A Dissertation submitted in part fulfillment for the

Degree of

Master of Medicine, Internal Medicine,

University of Nairobi.

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DECLARATION

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DEDICATION

To my wife Mariam whose relentless love has been an inspiration.

And

To my father Abdulsamad and mother Lutfunnissa for their sacrifices and unconditional support.

Finally

To my daughter Aliya who always filled my heart with joy.

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ACRONYMS

ART----------Antiretroviral therapy

BBB----------Blood brain barrier.

BHI----------Brain heart infusion

CFU----------Colony forming units

CLSI----------Committee of Laboratory Standard Institute

CNS----------Central Nervous System

CRAG----------Cryptococcal antigen

CSF----------Cerebrospinal fluid

CT Scan----------Computerized tomography scan

CVA----------Cerebro-vascular accidents.

EDTA----------Ethylene-diaminetetra-acetic acid

HAART----------Highly active anti-retroviral therapy.

HIV----------Human Immunodeficiency Virus

H_2O----------Water

ICP----------Intra-cranial pressure

IV----------Intravenous

KEMRI----------Kenya Medical Research Institute

Kg----------Kilogrammes

LP----------Lumbar puncture

MIC----------Minimal inhibitory concentrations

Mg----------Milligrammes

µg----------Microgrammes

ml----------Millilitre

µl----------Microlitre

mm----------Millimetres

NCCLS----------National Committee on Clinical and Laboratory Standards

Rpm----------Rotations per minute

SPSS----------Statistical Package for Social Sciences

ABSTRACT

Background: Cryptococcal meningitis caused by the environmental encapsulated fungus Cryptococcus neoformans, is an important and often fatal infection whose incidence has multiplied severally after the advent of HIV. Correct diagnosis and appropriate treatment is required to reduce the high mortality rates associated with it.

Objective: To determine the culture yield and the sensitivity of India ink as a diagnostic tool; To determine the sensitivity patterns of Cryptococcus neoformans to amphotericin B, fluconazole, miconazole and 5 flucytosine;and to determine the prevalent serotype of Cryptococcus neoformans at Kenyatta National Hospital.

Study design: Cross-sectional study.

Setting: In-patient medical wards, Kenyatta National Hospital, Nairobi, Kenya.

Study population: All patients admitted to medical wards of Kenyatta National Hospital with clinical diagnosis of meningo-encephalitis .

Study method: Patients with meningo-encephalitis were subjected to a lumbar puncture after excluding contra-indications. CRAG test was done on the CSF obtained. Positive CSF samples were subjected to India ink stain. The CSF was cultured and sensitivity patterns to amphotericin B, fluconazole, miconazole and 5 flucytosine were determined. Sero-typing of the isolates was carried out. The HIV status of the cases was determined.

Data entry and analysis: Data was entered into a computer database then analysed using SPSS version 12. Descriptive statistics were used for both continuous and categorical data. Inferential statistics were used to determine associations. The p value of <0.05 was considered significant.

Results: Three hundred and seven CSF specimens were obtained during a 3 month period. Sixty one tested positive for CRAG (19.8%).Three specimens

grew other fungi (*T.mucoides*-2 and*T.Beigelii*-1) on further microbiological analysis. Fifty eight specimens were thus analysed.

India ink was found to be positive in 33 CSF samples (56.9%); culture positive samples were 39 (67.2%).

Three isolates (7.7%) had MICs of >2µg/ml for amphotericin B and were thus resistant. Twenty eight isolates (71.8%) were susceptible to fluconazole having MICs of <4µg/ml; 8 patients (20.5%) had MICs of between 8 and 16µg/ml and were categorized as intermediate resistant; 3 (7.7%) were highly resistant having MICs of >16µg/ml. Miconazole susceptible isolates were 37(94.9%) having MICs of <1.0µg/ml; only 2 isolates had MICs >1µg/ml signifying resistance. 5-Flucytosine susceptible isolates were 19 (48.7%) having MICs <4µg/ml;13 (33.3%) patients had MICs of between 8 and 16µg/ml and were in the intermediate resistant group whereas 7 patients (17.9%) fell in the highly resistant category with MICs of >32µg/ml.

Fifty seven had underlying HIV and cryptococcus meningitis was the AIDS defining illness in 65.5% of them. One patient repeatedly tested negative for ELISA but this was not confirmed by a PCR.

All 39 isolates cultured were sero-type A (*Cryptococcus Neoformans var grubii*).

Conclusion: India ink that is currently being used widely as the sole diagnostic test for cryptococcal meningitis has a very low sensitivity thus misses a number of cases leading to misdiagnosis and inappropriate treatment decisions. The sensitivity patterns though concerning especially to Amphotericin B show that the isolates are still susceptible to the tested anti-fungals invitro.

INTRODUCTION

Cryptococcal meningitis caused by environmental encapsulated fungus is an important and often fatal infection if not appropriately diagnosed and treated. The HIV pandemic has raised the profile of cryptococcus neoformans from an obscure yeast to the most important fungal cause of morbidity and death worldwide. It's the most common opportunistic infection of the CNS in patients with AIDS and the most common cause of death in HIV infection after tuberculosis (1).Though generally associated with underlying HIV, other risk factors for this disease are: corticosteroid therapy, leukemia, lymphoma organ transplantation and connective tissue disease (2,3,4,5). Cryptococcal meningitis is also known to occur in immuno-competent individuals and apparently normal hosts (1,6,7,8).

The burden of cryptococcal meningitis especially in the developing world where access to HAART and optimal anti-fungal therapy is limited is high. In Zimbabwe cryptococcal meningitis constituted the most common cause of adult meningitis (21%). In these patients the median survival time was 14 days (9). In a cohort of HIV infected Ugandan adults cryptococcal disease was diagnosed in 77 out of 1372 individuals and was associated with 17% of all deaths (1).In Zambia where 230 patients with primary cryptococcal meningitis were studied, cryptococcal meningitis was the AIDS defining illness in 91%.There was a 100% case fatality rate observed in patients who received treatment with fluconazole monotherapy

and those who only received palliative care though the median survival from time of diagnosis was 19 days in the former and 10 days in the later group (10).

In the industrialized world despite the availability of HAART, cryptococcal meningitis continues to pose difficult management questions. Questions still remain regarding the optimal combination of anti-fungal agents, duration of treatment, accurate indications of response to therapy, management of raised ICP and role of adjunctive therapies such as corticosteroids or other anti-fungal agents.

LITERATURE REVIEW

Cryptococcus neoformans was first identified as a human pathogen in 1894 in Germany when it was isolated from the tibia of a patient by Busse and Buschke. The same year, it was isolated from peach-juice by Sanfelice (11).

The first description of Cryptococcal meningitis was published in 1905 by Von Hanseman although a case of chronic meningitis described in 1861 by Zenker prior to the pathogen isolation was probably the first case history (11).

Cryptococcus neoformans is an encapsulated round to oval yeast measuring 4 to 6 microns with a surrounding polysaccharide capsule ranging in size from 1 to over 30 microns when cultivated in laboratory (12). In its natural environment, it is smaller and poorly encapsulated. Mycelia are produced bearing basidiospores ranging from 1 to 8 microns. Given that particles less than 5 microns can enter the lung but only particles less than 2 microns can deeply penetrate the lung, it is postulated that transmission occurs via inhalation of the basidiospores or unencapsulated forms leading to colonization of airways and subsequent respiratory infections (13,14).

A recent study demonstrated that Cryptococcus neoformans could be isolated from the nasopharynx of approximately 50% of AIDS patients with Cryptococcosis whereas it was not isolated from AIDS patients without Cryptococcosis supporting inhalation as a portal of entry (15).

Although complement mediated phagocytosis is the primary initial defense against cryptococcal invasion, the absence of an intact cell-mediated response

results in ineffective ingestion and killing of the organism leading to dissemination and increased cryptococcal burden.

Given its antiphagocytic properties, the polysaccharide capsule composed mainly of glucoronoxylomannan is thought to be the organism's primary virulence factor. The exopolysacharides of the capsule may contribute to virulence by suppressing the immune response, inhibiting leukocyte migration and enhancing HIV replication (16).

Cryptococcus neoformans is distinguished from other yeasts by its ability to assimilate urea and it possesses membrane-bound phenoloxidase enzymes, which are able to convert phenolic compounds to melanin as demonstrated by certain agars such as birdseed agar. It is postulated that cryptococcus has a propensity to invade CNS because of its ability to synthesize melanin from catecholamines that are present in this tissue in large concentrations (17,18).

Cryptococcus has been considered a sporadic infection with a worldwide distribution. There are four serotypes of Cryptococcus neoformans designated as A, B, C and D based on antigenic determinants on the polysaccharide capsule (17). They have different environmental niches, geographical distribution and affect different patient groups. The vast majority of infections worldwide occur in HIV patients and are due to serotype A (*Cryptococcus neoformans var-grubii*) and Serotype D (*Cryptococcus neoformans var-neoformans*) (19). Serotypes B and C (*Cryptococcus Neoformans var-gatii*) are known to cause infection in immunocompetent but also in immunocompromised patients (20).

Cryptococcus grows readily from soil contaminated with avian excreta particularly those of pigeons possibly because the excreta are rich in xanthine, creatinine, urea and uric acid, all of which Cryptococcus can assimilate.

There have been no outbreaks attributable to environmental sources and no reports of animal to human transmission. Human to human transmission is rare. There is one case report of a person acquiring cryptococcal endolphthalmitis after receiving a corneal transplant and in another case; a health worker developed localized cutaneous cryptococcosis after auto-inoculation with blood from a patient with Cryptococcemia (17,21).

Substantial changes in the epidemiology of Cryptococcal meningitis have occurred with the evolution of AIDS epidemic. As the epidemic developed, there was marked increase but subsequent fall as a result of anti-fungals/HAART. Although presently, the overall incidence of cryptococcosis is unknown, it is higher among patients with AIDS in Africa and South East Asia than in U.S whereas it appears less frequently in Europe. In the developed world, the introduction of potent ARVs resulted in a decrease of opportunistic infections associated with AIDS. The incidence of life-threatening cryptococcal infections among patients with AIDS has been estimated at 6 to10% in the US, western Europe and Australia, and 15 to 30% in the sub-saharan Africa (22).

Although more males are reported to develop cryptococcal disease, the male to female ratio is essentially 1 when one corrects the female predominance in HIV infection.

Cryptococcossis in children with AIDS is less common with a prevalence rate of approximately 1.4% (23).

More than 75% of the cases associated with AIDS develop when the CD4 count falls below 50 cells/mm^3 (24). In 1996, a retrospective review of 65 AIDS patients with Cryptococcal meningitis in France, the median CD4 cell-count was 46 cells/mm^3 (25). Cryptococcal meningitis was the initial AIDS defining illness in 63%.In a study by Jowi et al, carried out in Nairobi Kenya, cryptococcus meningitis was the most common neurological complication of HIV comprising 22% of the total. The mean CD4 count was 60 cells/mm^3 (median 17,range between 1-273) (26).

A retrospective analysis of 326 clinically diagnosed cases with meningitis carried out over a period of five and a half years from Jan 1996 to June 2001 in India, found a prevalence of Cryptococcal infection to be 16.6% (54 patients). Of these, 28.9% (13 patients) had HIV infection, 20% (9 patients) were renal transplant recipients, 8.9% (4 patients) were diabetic and 2.2% (1 patient) had Systemic lupus erythematosus. In 40% (18 patients) no underlying disorder was found (27).

The impact of HIV infection on the spectrum of meningitis has been studied in South Africa where cryptococcal meningitis comprised 13% of all patients admitted with meningitis. It also constituted the fourth most common cause of meningitis after tuberculosis, acute bacterial meningitis and acute viral meningitis. All patients with cryptococcal meningitis were positive for HIV infection (28).

A larger study carried out in Rwanda between 1983-1992, involving 3476 CSF samples from 2824 adult patients with meningitis, found Cryptococcus to be the aetiological agent in 549 patients, (19%). It comprised the highest cause of meningitis followed by Neisseria, Streptococcus and tuberculosis (29).

A prospective observational study conducted over a 10-month period between 1st Jan and 31st Oct 1995 in Zimbabwe identified 89 patients from 406 in whom a clinical diagnosis of meningitis had been made, giving a prevalence of 21%. The median age was 34yrs and 56 patients were male (62%). Cryptococcus meningitis was the AIDS-defining illness in 88%, all of who were sero-reactive for HIV. The median CD4 cell count was found to be 70 cells/mm^3 (9).

A cohort of cryptococcal infection in HIV infected Ugandan adults done between Oct 1995-Jan 1999 found an overall incidence of 40.4/1000/year and it constituted the principal cause (most frequent) of meningitis in the cohort (1).

The epidemiological features point to Cryptococcal meningitis being a disease that is a common and an important contributor to morbidity and mortality especially amongst the HIV infected individuals. This is true especially across most of the sub-Saharan Africa where in the absence/unavailability of ARV therapy, care of HIV infected individuals is focused on infection prophylaxis and treatment of concomitant infections.

While meningitis is the most common manifestation of cryptococcal infection, other infection syndromes are well recognized.

Infection is believed to occur through inhalation and the primary site of infection is the lung. It is likely that most such infection is asymptomatic. Primary disease

may result in either immediate pulmonary or disseminated disease or it may be quiescent for many years with subsequent disease development following an immunosuppressive event. Disease has been described in almost all body systems but the brain remains the organ with a particular vulnerability to the infection.

Among immunosuppressed non-AIDS patients, CNS disease has also represented the most frequent clinical presentation of cryptococcal infection. Kaplan et Al reviewed autopsies of 23 cases of Cryptococcosis occurring from 1956 to 1972 at one of the largest cancer centers in the US. 87% of these cases had evidence of CNS infection (30).

Retrospective review by Husain et Al showed that of the 178 cases of cryptococcal infections in organ recipients, 72% had CNS infections (31).

After the beginning of the AIDS era, increase in the frequency of cryptococcal infections occurred and was mostly related to meningeal infections (32).

Different series of studies reported incidences of 67% (33), 76% (34) and 84% (35).

A recent comprehensive surveillance for Cryptococcosis by Hajjeh et al, revealed that 89% of HIV positive patients with this infection presented with meningitis or fungemia (36).

Cryptococcemia often precedes CNS invasion and may persist for an extended period of time (1 to 16 weeks) despite treatment (37). CNS invasion may be secondary to hematogenous infection or may represent reactivation of the disease similar to tuberculosis or histoplasmosis.

The time of onset of symptoms to diagnoses ranges from days to months (11), the longest duration recorded being 29 years (38).

Infection typically presents as a sub-acute process, however presentations characteristic of either acute or chronic meningitis can occur (38).

Most frequently presenting complaints include headaches, fevers, changes in levels of consciousness- somnolence, confusion, stupor or coma, dizziness, visual disturbances, seizures, unsteady gait, irritability, nausea or vomiting (35,38). Neck-rigidity seems to be uncommon in HIV occurring in about 25% (39) and focal neurologic signs in 20% (40).

The most common presentation being meningo-encephalitis (41), brain abcesses (cryptococcomas), isolated cranial-nerve lesions, subdural effusions, spinal cord lesions, dementia and ischemic stroke represent the extended spectrum of CNS disease.

Interestingly, clinical differences have been described depending on the infecting variety of Cryptococcus neoformans (42). When Cryptococcus neoformans var-gatii is the causative agent, papilloedema, pulmonary involvement, hydrocephalus and multiple enhancing lesions (cryptococcomas) on imaging studies are more frequent (43,44).

After an episode of cryptococcus meningitis, an important proportion of patients are left with permanent neurologic deficits. In the classic study by Diamond and Bennett, of the 62 cured patients, the ratio of sequelae were (45);

- Decreased mental functions (chronic brain syndrome) 31%
- Residual visual loss 8%

- Residual motor impairment 5%
- Residual cranial nerve palsies 3%.

A definitive diagnosis of cryptococcus meningitis requires lumbar puncture with demonstration of yeasts with Indian ink stain, positive cryptococcal antigen testing or culture of the organism. The inflammatory response caused by the Cryptococcus neoformans provokes the appearance of leucocytes in the CSF (50-500cells/μl). Although mononuclear cells are commonly predominant, neutrophilic pleocytosis, either at initial step or through the course of the whole illness occurs in upto 25% of cases (39). The level of pleocytosis is typically much lower in AIDS patients (38). Mildly increased CSF protein levels (<500mg/dl) related to host inflammatory response and to the presence of anti-cryptococcal antibodies are usually found (46).

At least one-fourth of patients with cryptococcal meningitis have decreased CSF glucose. This denotes a poor prognosis (38).

However, normal CSF parameters can also be found. This is reported in a study from South Africa which found that 17% of AIDS patients with cryptococcal meningitis had normal CSF parameters (47).

Microbiologic confirmation of Cryptococcus neoformans in CSF is the strongest proof of cryptococcus meningitis (48). From 149 HIV negative patients with cryptococcus meningitis, 89% were culture proven (48). When the underlying condition is AIDS, the rates of positive CSF cultures rise to 95 upto 100% (33,34).

India Ink is probably the most important screening test to rule out cryptococcal meningitis. The encapsulated cells of this yeast can be easily distinguished when using the black colloidal medium of this preparation. The test will be positive when about 10^3-10^4 CFU/ml, are present in the CSF sample. AIDS patients have larger concentration of yeast ranging between 10^5-10^7CFU/ml (38). In patients who are HIV negative, diagnoses of cryptococcal meningitis can be established in only 50% of the cases, whereas in patients with AIDS, yields can be up to 80% (38,48). Centrifuging the CSF to 500 rpm for about ten minutes can improve the sensitivity of this test (38). As useful as it can be for diagnostic purposes, the India Ink is not a good guide of response to therapy. Accordingly, Diamond and Bennett demonstrated that as long as the same sample is culture negative persistently, positive India Ink preparations did not correlate with poor outcome .Indeed India Ink remained positive for as long as eight years in one of their successfully treated patients(45).

A very useful test for diagnosis of patients with cryptococcal meningitis is the determination of CRAG titers in the CSF. This test has been consistently positive in over 90% of cases of cryptococcal meningitis among HIV patients. Various studies have reported sensitivities of 100%(33), 92%(34), 91%(35), 92.5%(49) and 98%(29).

While the CRAG test is very helpful, it is important to also keep in mind that false negative results may occur, particularly so when acapsular strains of Cryptococcus neoformans are isolated (50). Most false positive results are

caused by the presence of rheumatoid factor, which is eliminated by treating the specimen with pronase or dithiothreitol or boiling it with EDTA. Rarely false positive tests may occur when a cross-reactive antigen is present such as the polysaccharide of Trichosporon beigelii or another microorganism (51,52). Serum CRAG test is also very likely to be positive among patients with CNS Cryptococcossis particularly when compared with patients with disseminated non-neural Cryptococcossis (33). However, serum CRAG does not cross the Blood Brain Barrier and therefore blood levels do not have influence on CSF titers.

Importantly, CRAG tests are not recommended for follow-up and treatment decision because the kinetics of the antigen clearance both in CSF and serum are unpredictable (38).

A retrospective chart review evaluating the monitoring of serum CRAG titers in patients with AIDS related cryptococcal disease found that while 87% of 136 patients experienced a decrease in serum CRAG on treatment, no significant difference was found between serum CRAG tests of patients who had a clinical response to treatment and those of patients who experienced persistent disease, probable relapse or definitive relapse of cryptococcal disease. In fact 6 of the10 evaluable patients with evidence of a definitive relapse had a decrease in serum CRAG compared with previous titers (53).

The study by J.Bogaerts et al carried out in Rwanda also evaluated the predictive value of CRAG titers for culture outcome. At initial diagnoses, 53%{9/17}, 80%{82/103}, and 95%{218/229} of the CSF specimen showing CRAG titers of

<1:4, 1:4-1:128, >1:256 respectively had a positive culture for Cryptococcus neoformans (29).

In rare cases both India ink and latex for CRAG determination can remain negative despite the isolation of the organism by culture (50).

The relative insensitivity of each test means that none can be used solely as the gold standard for cryptococcal meningitis though from the above studies it is noted that CRAG determination is the most sensitive and specific of the three.

Molecular identification techniques combining high sensitivity and specificity will undoubtedly become a standard practice for the diagnosis of cryptococcus neoformans. However, these techniques will remain beyond the scope of a clinical laboratory in a developing country where the burden of cryptococcal infection is unfortunately the highest.

Other Findings

CSF opening pressures are usually high in patients with Cryptococcus meningitis ranging from 130mmH_2O to 310mmH_2O (54). This finding is more frequent for AIDS patients in whom upto 60% of cases may have pressures greater than 250mmH_2O and 30% of whom have pressures above 350mmH_2O (55,61). This parameter is considered a poor prognostic finding not only at the beginning of diagnoses but during the follow-up period.

Imaging of the brain is a critical management step for multiple reasons. First, cryptococcus meningitis related hydrocephalus may require surgical shunting for pressure relief and should be diagnosed early. Secondly, focal lesions require an imaging follow-up to determine if special therapeutic options are required. And

finally, there's always a possibility of second simultaneous process among AIDS patients requiring specific therapy (56).

A series that evaluated CT Scans in patients with cryptococcal meningitis found that 50% of patients with AIDS had a normal scan, 34% had diffuse cortical atrophy, 9% had hydrocephalus and 11% presented with focal masses (Cryptococcomas) (57).

In non-AIDS patients, CT Scan was normal again in 50% of the patients, 25% had hydrocephalus, 15% had gyri enhancement and 15-25% had focal masses, (single/multiple, both enhancing or non-enhancing) (58).

Cryptococcal meningitis is a serious condition with a rate of relapse ranging between 15-30% in HIV negative and no less than 50% in AIDS patients (38)

In AIDS patients, poor prognostic factors include;

- IV Drug Abuse (59)
- Age greater than 30years (25)
- Abnormal mental status (60)
- High opening CSF pressures of greater than 350mmH_2O (61)
- CSF findings of: low CSF glucose (25)
- Positive cultures at 2weeks (59)
- High titers of CRAG >1:32 (60,61)
- Positive blood and urine culture (62)

Most Cryptococcus neoformans isolates are initially sensitive to amphotericin B and those that acquire resistance do so during the course of therapy (63). There

has been concern over the possible emergence of fluconazole resistant cryptococci especially among AIDS patients who may require prolonged courses of anti-fungal therapy. Anti-fungal susceptibility would be useful in guiding selection and monitoring of anti-fungal therapy especially in cases of persistent or recurrent fungal infection. However, in-vitro, anti-fungal susceptibility testing is of questionable value because of limited correlation of results with clinical response (64).

Several reports in literature implying fluconazole resistance on the basis of clinical failure without laboratory evidence to support this conclusion, reflects the need for reliable and reproducible susceptibility testing (65).

In one study supporting the use of susceptibility testing, 28 isolates from 25 patients were tested for fluconazole susceptibility and correlated with clinical outcomes (66). Therapeutic failure was observed in 5 patients who were infected with isolates for which the fluconazole. Minimal Inhibitory Concentrations were greater than 16µg/ml. Of these 5 patients, 4 died from active cryptococcal disease. Of the 20 patients with fluconazole susceptible isolates, only 2 patients died because of causes unrelated to cryptococcal disease. These findings suggest that fluconazole susceptibility maybe useful in predicting clinical response. Another study suggests that fluconazole susceptibility might be useful for determining whether fluconazole can be used for primary therapy (67).

Most of the available data suggest that resistance is an unusual cause of relapse. An evaluation of 13 isolates from 5 patients with recurrent cryptococcal meningitis found the initial and relapse isolates to be clonally related confirming

that the recurrence was not from a new strain (68). There were no increases in the MICs among serial isolates tested with either amphotericin B or fluconazole, suggesting that relapse was not due to drug resistance but was probably secondary to host factors.

A similar larger study conducted by CDC investigators also concluded that recurrent disease was typically associated with relapse with the same strain, which remained susceptible to fluconazole (69).

Anti-fungal drug options for cryptococcal disease are limited. Amphotericin B is the main stay of treatment. It is fungicidal and in-vitro resistance is extremely rare, however nephrotoxicity is a significant problem although it is usually reversible if the total dose does not exceed 4 gms (70). It has to be administered intravenously as oral bio-availability is poor. It causes membrane disruption through binding to sterols in the cell membrane but also probably has an effect through stimulating macrophage function. Lipid formulations have the advantage of lower toxicities and can be given in higher doses of upto 10mg/kg/day but are considerably more expensive (71).

5 Flucytosine is a nucleotide analogue and appears to have a synergistic action with Amphotericin B in-vitro (72). A randomized trial showed a trend towards more rapid CSF sterilization in patients receiving 5 flucytosine in combination with amphotericin B compared to amphotericin B alone (59). It has the disadvantages of cost, poor tolerability, rapid development of resistance if used as monotherapy and the need to monitor blood-levels.

The azole drugs have revolutionalized the treatment of fungal infections because of their potency, tolerability, good CSF penetration and oral/Intravenous formulations. There is more experience with fluconazole in the treatment of cryptococcal meningitis. While it is not as potent as itroconazole in-vitro, it has better CSF penetration and appears more effective in clinical trials (73). The newer azoles such as voriconazole and posaconazole appear to have better in-vitro activity against cryptococcus neoformans than fluconazole but there is no data from controlled trials on cryptococcal meningitis.

Current treatment guidelines separate treatment into three phases (74). In the induction phase lasting for two weeks, Amphotericin B at a dose of 0.7-1.0mg/kg/day intravenously combined with 5 flucytosine at a dose of 100mg/kg/day in four divided doses is administered. This is followed by a consolidation phase using fluconazole at a dose of 400mg/day for 8-10weeks. The maintenance phase consists of fluconazole at a dose of 200mg/day to be continued lifelong but can be stopped after immune reconstitution with HAART where the CD4 counts are above 200cells /µl (75).

In sub-saharan Africa including Kenya there is unavailability of 5 flucytosine, thus the induction phase consists of Amphotericin B at the same dosage and duration. There need to be more treatment trials in cryptococcal meningitis but with the advent of HAART, there is a decreasing interest in this disease in industrialized countries, though the same cannot be said for developing countries where due to poor resource settings, there remains an unavailability of HAART and first-line anti-fungals (10).

STUDY JUSTIFICATION

Cryptococcus meningitis is a significant public health burden in developing countries where it constitutes the most common cause of adult meningitis in HIV patients. In addition high rates of mortality are observed with and without specific treatment.

There have been a number of studies, both regionally, and worldwide on various aspects of the disease but very few have been done in Kenya.

India ink is currently the sole diagnostic tool in many Kenyan hospitals due to its availability and low cost. Its low sensitivity means that a number of cases of cryptococcal meningitis are left undiagnosed and hence inappropriately treated. Thus it would be useful to know its utility.

There has been a concern over the possible emergence of fluconazole resistant cryptococci especially among AIDS patients thus anti-fungal susceptibility would be useful in guiding selection and monitoring of anti-fungal therapy.

Serotype B and C are known to be more virulent than serotypes A and D requiring prolonged treatment thus the need to know the prevalent sero-type in Kenya so as to optimize the current anti-fungal regime.

OBJECTIVES

BROAD OBJECTIVES:

To determine the sensitivity patterns, serotypes of Cryptococcus neoformans and diagnostic value of India ink stain in patients with Cryptococcal meningitis at Kenyatta National Hospital.

SPECIFIC OBJECTIVES:

1.To determine the relative sensitivity of India ink in patients with Cryptococcal meningitis .

2.To determine the culture yield of Cryptococcus neoformans in patients with Cryptococcal meningitis .

3.To determine the sensitivity of Cryptococcus neoformans to amphotericin B, fluconazole, miconazole and 5 flucytosine.

4.To determine the prevalent sero-type of Cryptococcus neoformans in the study group.

5.To determine the HIV status of patients with Cryptococcal meningitis.

MATERIALS AND METHODS

Study Design: Cross- sectional study.

Study Area: Kenyatta National Hospital, medical wards.

Study Population: Patients with meningo-encephalitis who were CRAG positive on CSF studies.

Sample size

CI- 95%

d- absolute precision 5%

P-95% (predictive value for culture outcome in Rwanda)

$Z^{1-\alpha}$ standard error of mean =1.64

n= sample size

$$n=\frac{Z^2 x P\ (1-P)}{d^2}$$

n=51

Sampling Technique: Consecutive patients with clinical diagnoses of meningo-encephalitis getting admitted to medical wards were screened for CRAG positivity in CSF till the required sample size was reached.

Inclusion Criteria.

1 Patients admitted to the medical wards of KNH with clinical features of meningo-encephalitis including acute, sub-acute and chronic presentations.

2 Patients who were CRAG positive in CSF.

Exclusion Criteria.

1 Patients in whom lumbar puncture was contraindicated:

- Patients with papilloedema on fundoscopy.
- Patients with focal neurological deficits.
- Patients with space occupying lesions confirmed by CT-Scan.

2.Any patient or guardian who declined to give consent.

3.Patients with documented proof of prior treatment for cryptococcal meningitis with Amphotericin B.

METHODOLOGY

Clinical Methods:

On the post admission days of the respective medical wards patients who had been admitted with a clinical diagnosis of meningo-encephalitis were re-evaluated by the investigator. A complete medical history was taken and a physical examination including a fundoscopy was carried out. Those patients in whom an LP was not contraindicated were subjected to one after consenting

CSF collection:

CSF was collected from the subarachnoid space maintaining aseptic techniques using a sterile wide bore needle (gauge 21) inserted between the 4^{th} and 5^{th} lumbar vertebrae. Approximately 5mls of CSF was collected. Four mls was put into a sterile specimen bottle and taken to the KNH microbiology laboratory for the study purpose (76).One ml was put in a fluorinated bottle and taken to the biochemistry lab for routine proteins and glucose levels.

Laboratory methods:

A CRAG test was done on the CSF obtained using the Remel kit (Lenexa-USA) according to the manufacturers protocol. The CSF samples which tested positive were subjected to an India ink using standard laboratory procedures. It was then cultured into a soya enriched media and later sub cultured into the Sabarouds media. Positive cultures were inoculated into distilled water vials and were taken to KEMRI where they were purified and a biochemical analysis using API Candida was carried out to differentiate other yeasts. Sensitivity patterns of the isolates that were confirmed to be Cryptococcus Neoformans were determined

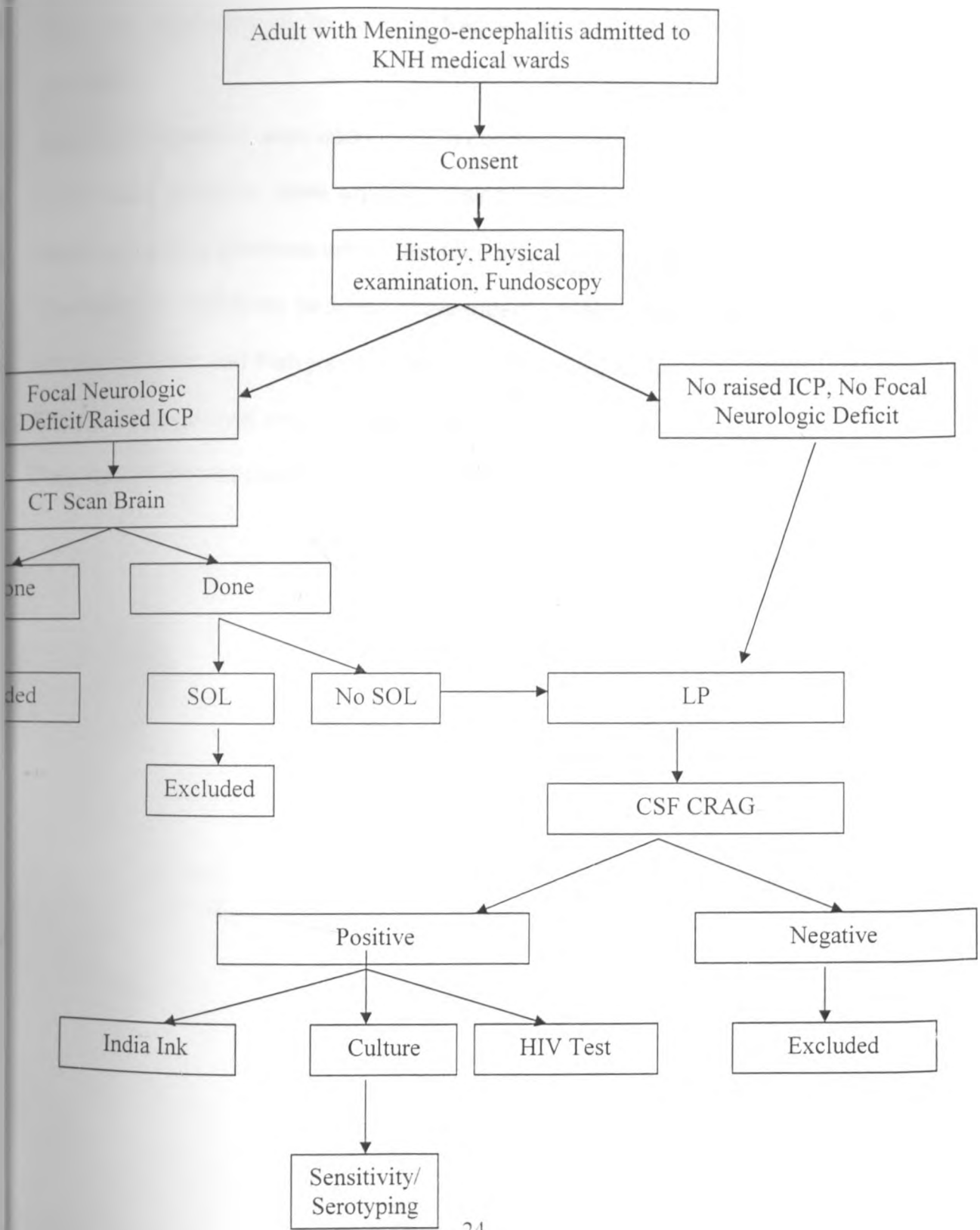
following the CLSI broth dilution protocol (77). Anti-fungal resistance was defined as follows: MICs of >2µg/ml, >16µg/ml, >1µg/ml, >16µg/ml for Amphotericin B, fluconazole, miconazole and 5 flucytosine respectively.

The MICs of fluconazole and 5FC between 8 and 16µg/ml were further categorized as Intermediate resistant or Susceptible Dose dependent (SDD). An inhouse strain of fully susceptible Cryptococcus neoformans was used as an internal control for reproducibility of the procedures. Sero-typing was carried out using the crypto-check agglutination kit.

An HIV test using ELISA 1 and 2 (Virunostica HIV Uni-Form II Ag/Ab; The Netherlands) was done on a blood specimen obtained after appropriate councelling at the immunology lab KNH.

Figure 1:

Flow chart presentation of methodology.



Data analysis and presentation:

Data was recorded on a proforma, entered into a database and analysed using SPSS version 12.0.

The data was cleaned by running frequencies and all missing entries were corrected.

Descriptive statistics were used for both continuous and categorical variables.

Continuous variables were analysed using measures of central tendency and dispersion and proportions were used for categorical variables.

The levels of sensitivity between independent variables were determined using Chi square tests and Fishers exact test in case of 2X2 tables.

The significance level was interpreted at p 0.05.

Data was presented using tables charts and histograms.

ETHICAL CONSIDERATION

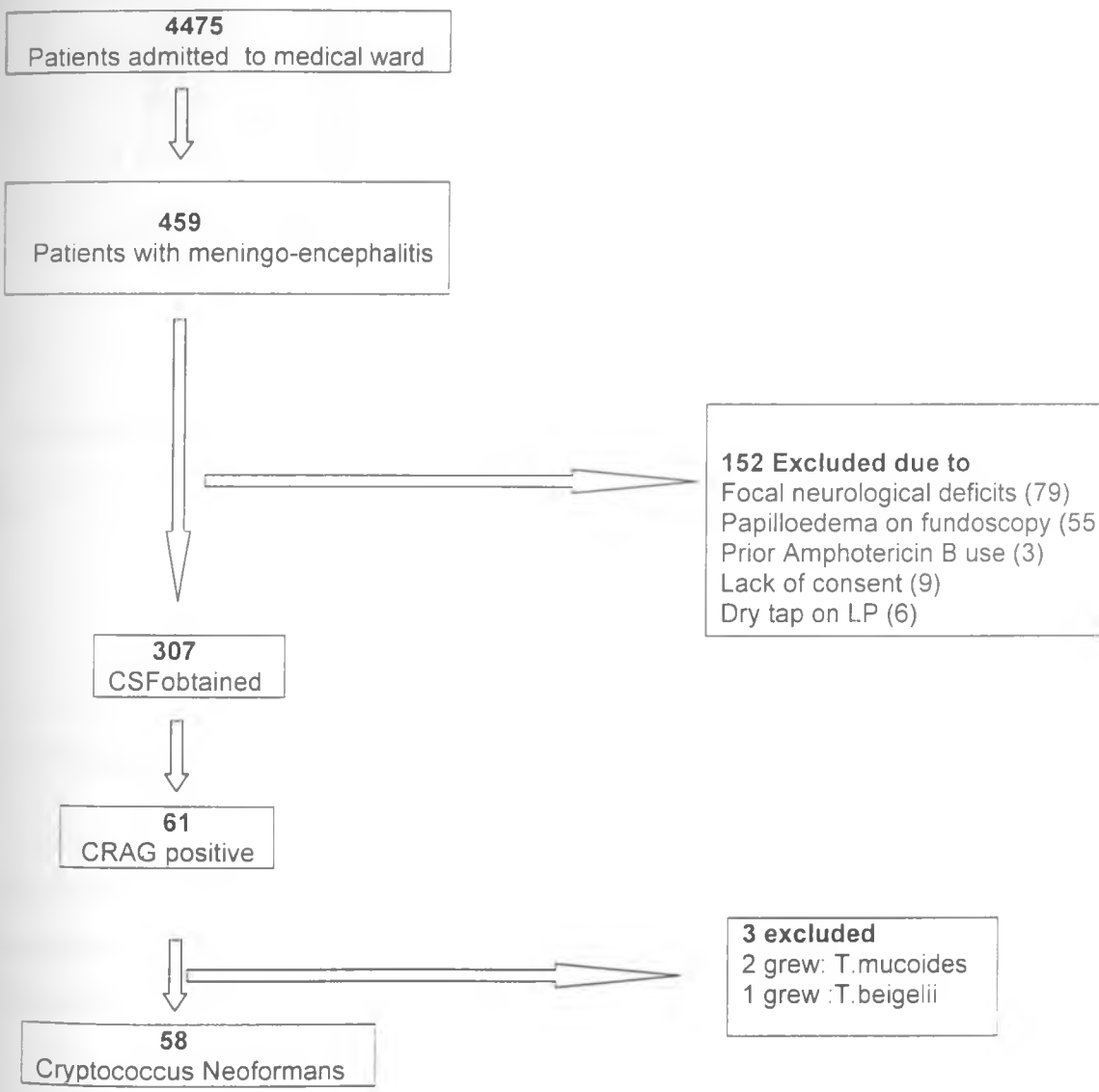
The Ethical Research Committee-KNH granted permission to carry out the study on 8th May 2006 after The Department of Medicine, University of Nairobis' approval. Patient's identities were kept confidential. Participation was voluntary. Inform-consent for inclusion in the study was obtained from the patients or if too ill to give consent, was taken from the guardian/ next of kin. Pre-test and post-test counseling for HIV test was done. The entire procedure was carried out maintaining strict aseptic techniques. Results of the study were relayed to the primary physicians taking care of the patients to help them make appropriate management decisions. CSF samples of patients who had been excluded were sent for other routine investigations as required or requested by their respective ward supervisors. A follow up of these patients was done by the principal investigator to ensure optimum care possible.

RESULTS

RECRUITMENT

Recruitment into the study began on 30th May 2006 and ended on 28th August 2006 (91 days). A total of 4475 patients were admitted to the medical wards during this period. Meningoencephalitis comprised 10.25% (459 patients) of the total admissions. Cerebrospinal fluid was obtained in 307 patients out of whom, through consecutive sampling, 61 were positive for CRAG. Fifty eight CSF specimens were included in the final analysis as shown in the recruitment profile below (figure 2).

Figure 2:



DEMOGRAPHIC DATA

The mean age of the patient population was 37.22 years (95% CI 34.83-39.62) with a range of 20-63 years (Fig.3). There were 25 males (43.1%) and 33 females (56.9%). The distribution of the marital status is shown in *Table 1.*

Fig 3:

Age distribution of patients with Cryptococcal Meningitis(n=58).

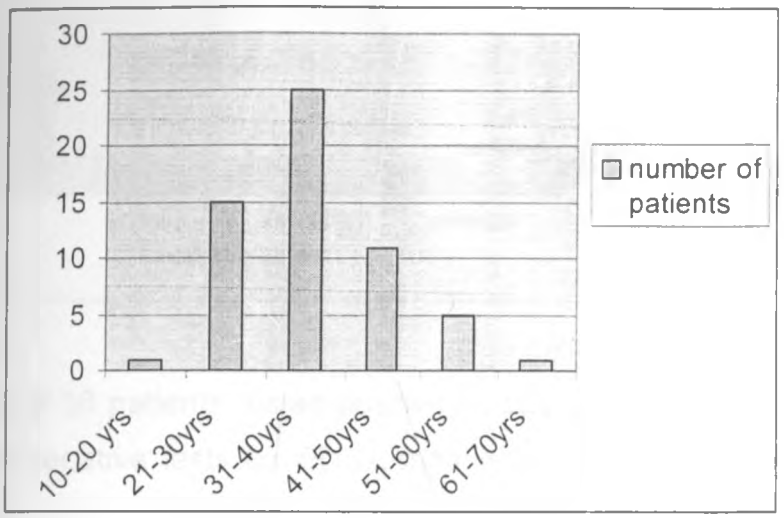


Table 1:

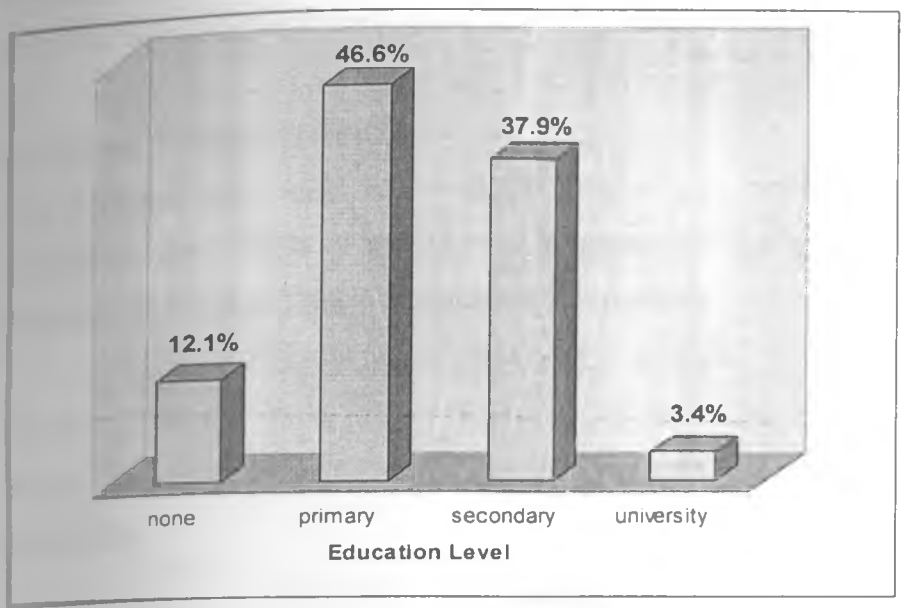
Marital status of patients admitted with Cryptococcus meningitis

Marital status	Frequency	Percentage (%)
Single	9	15.5
Married	41	70.7
Divorced	6	10.3
Widow	2	3.4

Most of the patients had at least a primary education (84.5%) whereas university graduates comprised 3.4% of the patients. The most common occupation was that of a housewife comprising of 22.4% of the total patients and 40% of the females.

Fig 4:

Education level of patients with cryptococcal meningitis



HIV STATUS

Fifty seven out of 58 patients tested positive for HIV (ELISA 1 and 2), 1 patient had repeatedly negative tests for ELISA though a confirmatory test (PCR) was not done.

Nineteen patients (32.8%) knew of their positive status before admission out of which 16 (27.6%) were already on HAART; 10 of them in their first 2 months of treatment. Cryptococcus meningitis was an AIDS defining illness in 38 out of the 57 (65.5%) patients. (*Table 2*)

Table 2:

HIV status of patients with Cryptococcus meningitis and HAART

Variable	Frequency	Percentage
HIV status		
Known	19	32.8
Newly diagnosed	38	65.5
Negative	1	1.7
HAART		
Yes	16	28
No	41	72

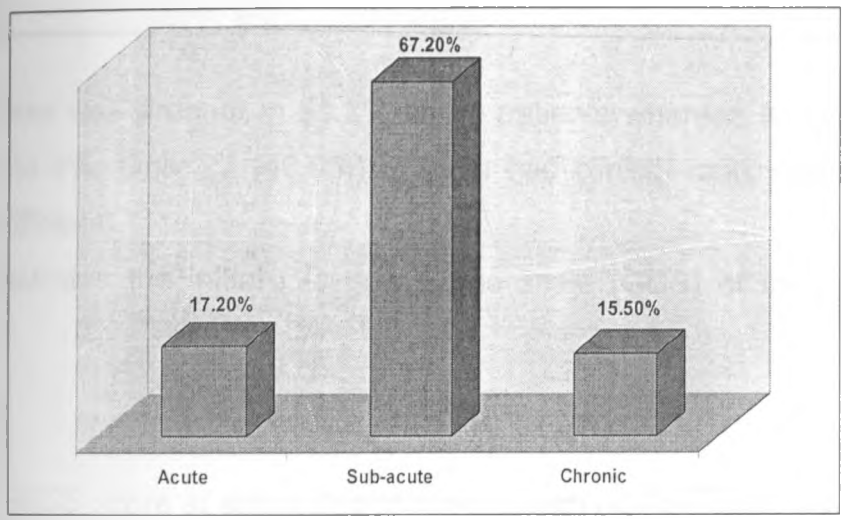
The most common associated illness was tuberculosis in 39.7% of the patients. Generalised lymphadenopathy was present in 17 (29.3%) whereas oro-pharyngeal candidiasis was noted in 36 (62.1%) patients.

CLINICAL PRESENTATION

Fig 5 shows the mode of onset of symptoms in patients with Cryptococcus meningitis, and *Table 3* shows the symptomatology of these patients where headache constituted the most common complaint.

Fig 5:

Mode of onset of symptomatology of patients admitted with Cryptococcus meningitis



Acute:<7 days, Subacute:7-28 days,Chronic:>28 days

Table 3:

Prevalence of presenting complaints of meningoencephalitis in Cryptococcus meningitis

Complaint	Count	Percentage (multiple outcomes)
Headache	48	82.8
Vomiting	41	70.7
Visual disturbances	38	65.5
Nausea	34	58.6
Fever	28	48.3
Confusion	25	43.1
Unsteady gait	22	37.9
Dizziness	19	32.8
Somnolence	4	6.9
Stupor	4	6.9

Neck stiffness was present in 55.2% of the patients whereas Kernigs sign was elicited in 34.5%. Only 27 (46.6%) patients had clinical meningism (headache and neck stiffness).

Table 4 illustrates the initial Glasgow Coma scale (GCS) of these patients at admission.

Table 4:

GCS score at admission of patients with cryptococcus meningitis

GCS	Frequency (%)
4-8 (comatose)	4 (6.9)
9-13 (semicomatose)	20 (34.5)
14-15 (alert)	34 (58.6)
TOTAL	58 (100)

LABORATORY

1.INDIA INK SENSITIVITY

India ink was found to be positive in 33 patients (56.9%). It was also found to be false positive in the two fungi (T. Mucoides, T.beigelii).

Table 5:

India ink sensitivity in Crag positive patients with Cryptococcus meningitis

India Ink	Frequency (%)
Positive	33 (56.9)
Negative	25 (43.1)
TOTAL (CRAG+)	58 (100)

2.CULTURE OUTCOME

Of the 58 specimens that were CRAG positive 39 grew Cryptococcus Neoformans (67.2%).

Table 6:

Culture outcome in Crag positive patients with Cryptococcus meningitis

Culture outcome	Frequency (%)
POSITIVE	39 (67.2%)
NEGATIVE	19 (32.8%)
TOTAL CRAG (+)	58

Table 7:

India Ink vs culture outcome of patients with Cryptococcus meningitis

		India ink		
		Positive	negative	total
Culture	positive	29	10	39
	negative	4	15	19
		33	25	58

The PPV of India ink for culture outcome was 74.36% whereas the NPV was 78.95%.

3 SENSITIVITY PATTERNS:

Table 8 summarises the susceptibility of Cryptococcus Neoformans to various antifungals.The geometric mean (GM) MICs, modes, ranges, MICs including 50% of isolates (MIC_{50}) and MICs including 90% of isolates (MIC_{90}) of the antifungal agents tested are shown in *Table 9*.

Thirty six (92.3%) Cryptococcal isolates were sensitive to amphotericin B with MIC's of less than 2µg/ml.

Fluconazole exhibited a less potent activity in-vitro with MIC_{50} of 2 µg/ml and MIC_{90} of 8µg/ml. Highly resistant cases comprised 3 patients (7.7%) who had MIC's of >16µg/ml.

Thirty seven isolates (94.9%) were sensitive to miconazole (MIC's of < 1µg/ml) whereas only 2 (5.1%) were resistant (MIC's of >1µg/ml).

5 Flucytosine was the least potent of the anti-fungals. MIC_{50} was 4µg/ml where as MIC_{90} was 16µg/ml. Only nineteen isolates (48.7%) were sensitive to the drug (MIC's of <4µg/ml).

Table 8:

Susceptibilities of cryptococcus neoformans to antifungals

DRUG	SENSITIVITY STATUS n(%)		
	Sensitive	Resistant	Highly resistant
Amphotericin B (n=39)	36 (92.3)	0	3 (7.7)
Fluconazole (n=39)	28 (71.8)	8 (20.5)	3 (7.7)
Miconazole (n=39)	37(94.9)	0	2 (5.1)
Flucytosine (n=39)	19 (48.7)	16 (41.05)	4 (10.25)

Table 9:
Susceptibility data (in µg/ml) of 39 clinical isolates of Cryptococcus Neoformans

Antifungal agent	MIC_{50}	MIC_{90}	Range	Mode	Geometric mean	No(%) of strains with antifungal resistance
Amphotericin B	0.125	0.5	0.03-8	0.125	0.170	3 (7.7)
Fluconazole	2.0	8.0	0.125-32	2.0	1.645	3 (7.7)
Miconazole	0.25	0.5	0.05-2	0.5	0.205	2 (5.1)
5 Flucytosine	4.0	16.0	0.125-64	8.0	4.611	4 (10.25)

MIC_{50} MIC value causing inhibition of 50% of isolates; MIC_{90},MIC value causing inhibition of 90% of isolates.
Antifungal resistance was defined as MICs of Amphotericin B>2µg/ml; MICs of fluconazole>16µg/ml; MICs of miconazole>1µg/ml; MICs of 5FC>32µg/ml.

4.SEROTYPES

Only serotype A was identified in all the 39 specimens that were subjected to the agglutination test.

DISCUSSION

Before the AIDS era Cryptococcosis was a well recognized but rare disease worldwide (11). It has become the most frequent cause of adult meningoencephalitis in patients with HIV which if not appropriately diagnosed and treated is invariably fatal (9).
This study was carried out at Kenyatta National Hospital, a tertiary referral center that also provides the facilities for direct access to medical care.

In this study 58 patients out of 307 (19.8%) in whom a CSF was done were diagnosed with Cryptococcal meningitis. Cryptococcus neoformans was the most common organism isolated. This is comparable to studies done elsewhere for instance in Rwanda, Zimbabwe and South-Africa prevalences of 19% (29), 21.9% (9) and 13% (28) were noted. In Rwanda and Zimbabwe cryptococcal meningitis constituted the most common cause of adult meningitis whereas in South Africa it constituted the fourth most common cause after tuberculosis, acute bacterial meningitis and acute viral meningitis.In the later 2 countries all patients had underlying HIV as a risk factor whereas the Rwandan study did not evaluate the serostatus of the patients.

The mean age of the patients in the study group was 37.22 years (38.8 for males and 36.6 for females). This is in line with the AIDS prevalence data where most patients are of the reproductive ages of between 15 and 49 (78). The male to female ratio was 1.5 to 2.

In this study Cryptococcus meningitis was the AIDS defining illness in 65.5% of the patients. Regionally the rate has been higher; 88% in Zimbabwe (9), 84% in South Africa (28) and 91% in Zambia (10). In USA it was the AIDS defining illness in 45% (32). This could be attributed to recognition of HIV at earlier stages before the CD4 drops to below 100 cells/mm^3. Out of the 19 patients known to have HIV disease, 16 were already on HAART; 10 of them in their first 2 months

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of treatment (ARVs). This could either be due to unrecognized disease at the initiation of HAART or by the development of the Immune Reconstitution Syndrome (IRS) in these patients where an exaggerated response to untreated opportunistic infections including Cryptococcosis occurs. Only 1 patient tested repeatedly negative for HIV on ELISA. She had been admitted for a pleural effusion, nephritis and confusion episodes. This patient eventually died and in the investigators opinion could either have been in the post zone phenomenon where there was a total antibody wash out thus the ELISA negativity; or could have been having an underlying connective tissue disease that was not investigated for and which could have predisposed her to infection with Cryptococcus neoformans.

Cryptococcal antigen (CRAG) is a highly sensitive antigen detection test for diagnosis of Cryptococcus neoformans thus it was used as the goldstandard. Only very rarely would uncapsulated cryptococcus neoformans be isolated through culture where CRAG is negative. False positives due to cross reactivity of antigens have been known to occur (51, 52). In this study, 3 isolates grew fungi other than Cryptococcus neoformans (2 *T.Mucoides*, 1 *T.Beigelii*). These organisms are quite resistant to standard anti-fungals and have an 80-100% mortality. It could explain why some of the patients diagnosed as Cryptococcal meningitis based on CRAG/ India Ink do not perform well despite aggressive and optimal approaches to the disease.

India ink is a relatively cheap, operator dependent and whose sensitivity increases with centrifugation of the specimen. It is the most widely used method in most parts of Kenya, and the only available tool to diagnose Cryptococcus meningitis. In this study despite the centrifugation of the specimen, sensitivity of India ink was found to be 56% taking CRAG as the gold standard of diagnosis. This was remarkably low compared to other studies where it was 85% in Zimbabwe (9), 83% in Rwanda (29) and 72% in New Zealand (43) where the gold standard was taken as a CRAG and/or a culture positive. It could be

attributed to possibly a low burden of the yeast in the CSF. Prior anti-fungal use may also have contributed to the low detection. India ink was also found to be positive in the two other fungi (*TMucoides* and *T.Beigelii*) suggesting that it is not 100% specific for Cryptococcus neoformans.

Culture yield was relatively low compared to other studies. Whereas in most studies patients with AIDS have yields of above 80% (9, 29, 43), in this study it was only 68%. This could have been due to various reasons. In a study done in Rwanda, culture outcome correlated well with CRAG titers. In patients with titers of < 1:4, yield was 53% compared to titers of > 1:256 where the yield was 95% (29). Determination of the CRAG titers was not part of this study but it could be postulated that titers were low at initial presentation. Prior anti-fungal use could have contributed to the low yield. Ten patients had a history of fluconazole use out of which 3 patients did not grow any organism on culture.

High rates of mortality, fungal persistence and frequent disease relapses have sparked a growing concern among clinicians regarding the potentials for the emergence of anti-fungal resistance among Cryptococcus neoformans. Resistance to amphotericin B has been scarcely reported both regionally and worldwide (79, 80, 81). A study in Spain reported resistance of 5.3% but it did not report whether this was primary or secondary (82). There have been few isolated reports of secondary amphotericin B resistance in patients with relapses of the disease (43). The finding of 3 isolates (7.7%) with amphotericin B resistance in patients who had no prior exposure to the drug could be alarming. The only evidence of its possible emerging resistance comes from a study that compared isolates from Malawi, U.S.A. and Thailand. In this study the mean MICs for isolates from Malawi were found to be 1.6µg/ml whereas they were 1.4µg/ml in the U.S.A (83). However in the present study the MIC_{90} for Amphotericin B was 0.5µg/ml and this was well below the cut of for resistance signifying that it should remain the drug of choice.

Fluconazole resistant isolates comprised 7.7%. These results are comparable to other studies for instance resistant isolates comprised 4.2% in Malaysia (81), 5.3% in Spain (82), 3.8% in Australia (43) and 8% in the U.S.A (84). Regionally 6% resistance was documented in Malawi (83), 5% in Uganda (85) and 11.2% in Kenya (86). Of the 3 patients who were resistant, history of prior fluconazole use was elicited in 2 of them. One patient denied usage of any anti-fungals prior to admission. This implies the possible existence of both primary as well as secondary resistance in Kenya. Secondary resistance to fluconazole that is currently a major concern especially in sub Saharan Africa can be attributed to poor prescription practices, sub therapeutic regiments, unavailability of alternatives such as the newer azoles (voriconazole, posaconazole, rovuconazole) and lack of laboratory capacity of skilled personnel for susceptibility testing. There have been reports of apparent improvement in susceptibility of Cryptococcus neoformans to fluconazole (79). This may reflect in part to a decrease in the overall drug pressure due to these agents concomitant with a decrease in Cryptococcosis among HIV infected individuals receiving HAART. On the contrary other studies note a decrease in susceptibility to the same drug and this has been attributed to prolonged use of fluconazole as prophylaxis together with HAART (80).

Susceptibility testing to 5 flucytosine has yielded mixed results. Whereas there was nil resistance of the isolates to the drug in Malawi, Thailand and U.S.A. (83) other studies have documented resistance of 32% (Latin America) (79), 8.3% (Malaysia) (81) and 21.2% in isolates from Kenya (86). In the present study 5 flucytosine resistance was detected in 10.25% of the patients. This implies that the epidemiology and drug susceptibility of Cryptococcus neoformans strains from some African countries as well as western countries are different and there could be possible existence of pockets of resistance.

Miconazole susceptible isolates (MIC of < 1µg/ml) comprised 94.9% whereas the resistant cases were only 5.1% (MICs of > 1µg/ml). Miconazole has poor

penetration into the CSF and despite its high sensitivity cannot be used in Cryptococcus meningitis.

Serotype A (*Cryptococcus Neoformans Var Grubii*) was the only sero-type identified in this study. This is in consistence with the other reports of the worldwide distribution of this sero-type and its predilection for HIV/AIDS (29,43,80). The presence of eucalyptus trees in Kenya and the tropical climate favour the presence of Serotype B and C. However these Serotypes were not identified in the present study group. There could be 2 reasons for this; Firstly Serotypes B and C are more predominant in immunocompetent individuals. Secondly, patients with these serotypes present more with focal neurological deficits, papilloedema, hydrocephalus and Cryptococcomas. These patients were excluded from the study.

CONCLUSION

Nearly half of the cases of Cryptococcus remain undiagonised if India ink is solely used for diagnosis. Three isolates which were both CRAG and India ink positive, grew other fungi (*T.Mucoides* -2, *T.Beigelii* –1).

This study confirms the emergence of strains of Cryptococcus neoformans that are primarily resistant to Amphotericin B; however 90% of the isolates were inhibited by MICs of 0.5µg/ml suggesting its highly potent activity thus should remain the drug of choice in treatment. Only 3 isolates (7.7%) were highly resistant to fluconazole supporting its continued use.5 Flucytosine is a very toxic drug and the evidence of its primary resistance should influence clinicians to avoid it in the induction phase of treatment of Cryptococcus meningitis in our set-up.

Only serotype A (*Cryptococcus neoformans Var Grubii*) was isolated in all the 39 patients.

LIMITATIONS:

1. CRAG was used as the Gold standard.
2. A number of patients with lateralising signs, papilledema, were excluded.
3. CD4 count and viral loads were not done.
4. CRAG titers were not determined.

RECOMMENDATIONS

1. India ink should not be used as the sole diagnostic test for cryptococcal meningitis and more sensitive tests such as CRAG be adopted countrywide for diagnosis of Cryptococcus meningitis.

2. Formulations of policies for the use of anti-fungals in management as well as prophylaxis in HIV patients.

3. Further studies to correlate in-vitro susceptibilities of the organism to the clinical outcomes.

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APPENDIX I:CONSENT FORM

Purpose of the study: Is to determine the sensitivity patterns and serotypes of the organism in patients with Cryptococcal meningitis.

Procedure: It involves you or your next of kin answering a questionnaire where details of your medical history will be noted. Routine physical examination and a lumbar puncture that involves obtaining a fluid sample from the lumbar spine will be carried out after which relevant laboratory tests will be undertaken on the sample obtained.

In case the laboratory test (CRAG) is positive you will be pre-test and post test counseled to have an HIV test done.

Benefits: This study will help in effective management of your condition and will also lay a basis for future treatment policies that may benefit other patients suffering from the same disease.

Risks: There will be no additional risks involved since there will be no other invasive procedures and you will not be actively participating in the study.

Participation: Participation is voluntary.

Costs: There will be no additional costs for the laboratory tests further required by the study.

Confidentiality: Your identity, sero-status and results of the study will be kept confidential.

CONSENT FORM

I _____ hereby consent to participate in the proposed research on cryptococcal meningitis sensitivity patterns and serotypes.

I am aware that the study involves a clinical examination and laboratory investigations on the cerebrospinal fluid obtained from my lumbar spine.

I agree to be tested for HIV the implications having been explained to me.

I understand that my identity and results of the investigation will be kept strictly confidential.

I have also been explained the benefits of this study for myself and others suffering from the same condition.

Signature of participant / next of kin. Date

_____ _____

Signature of Investigator

_____

Contact of Investigator: 0722370986.

APPENDIX II:BUDGET

PROPOSAL DEVELOPMENT		
50 hrs of literature search (internet)@Ksh 1 per minute		3000
Stationary		2000
Typing and printing proposal@ Ksh 25		5000
Photocopying proposal, questionnaires, data collection forms,		
Consent forms @Ksh3		5000
Binding of proposal (10 copies)@ Ksh 100		1000
SUB-TOTAL		16000
DATA COLLECTION		
Transport		5000
MATERIALS	AMOUNT	COST
Sterile gauze	300	3000
Sterile gloves	300	3000
Betadine solution	1 litre	1000
Lumbar puncture needles	300	3000
CSF specimen bottles	600	6000
CRAG kits	10	160000
India ink	60	6000
ELISA for HIV	60	12000
Culture sensitivity	60	12000
Serotyping	60	12000

SUB-TOTAL	218,000
THESIS WRITE UP	
10 hrs of literature search @K sh 1	500
Typing and printing of thesis@Ksh 25	5000
Photocopies of thesis @K sh 2 per page	2000
Binding of thesis 10 copies @K sh 250	2500
SUBTOTAL	10000
TOTAL	249,000

APPENDIX III: TIME FRAMEWORK

Proposal development	Nov 2005 to March 2006
Ethical approval	April 2006 to May 2006
Data collection	May 2006 to Aug 2006
Data analysis	Sept 2006 to Dec 2006
Thesis write-up	Jan 2006 to March 2007

APPENDIX IV: STUDY PROFORMA

Investigator: Date:

PERSONAL HISTORY

Patients Name: Study no:

IP No: Ward:

Sex (M=1,F=2) ( )

Age (yrs) ( )

Residence:

Occupation:

MEDICAL HISTORY

HIV status (negative=0,positive=1) ( )

CD4 count: (Unknown=0,Known=1) ( )

Other underlying diseases:(No=0,Yes=1) ( )

Tuberculosis: ( )

Malignancies: ( )

Diabetes: ( )

Renal disease: ( )

Connective tissue diseases: ( )

Transplant recipient: ( )

Previous admissions:(No=0,Yes=1) ( )

Cryptococcal meningitis ( )

Drug History:(No=0,Yes=1)

HAART ( )

Amphotericin B ( )

Fluconazole ( )

Steroids ( )

Immunosuppressants ( )

Septrin ( )

Presenting Complaints: (No=0,Yes=1)

Headache ( )

Fevers ( )

Somnolence ( )

Stupor ( )

Confusion ( )

Coma ( )

Dizziness ( )

Visual disturbances ( )

Unsteady gait ( )

Irritability ( )

Nausea ( )

Vomitting ( )

Duration of symptoms:

<1 week – acute=1

1 to 4 weeks- sub acute=2

>4 weeks-chronic=3 ( )

PHYSICAL EXAMINATION

Absent=0,Present=1

Neck stiffness ( )

Kernigs sign ( )

Lymphadenopathy ( )

Candidiasis ( )

GCS Score ( )

GCS score range:3 –8:Severe coma (1)

9-13:Semicomatose (2)

14-15:Alert (3) ( )

CSF RESULTS

India ink (negative=0,positive=1) ( )

Culture (Negative=0,Positive=1) ( )

Sensitivity:(Sensitive=1,Resistant=2,Highly Resistant=3)

Amphotericin B ( )

Fluconazole ( )

Miconazole ( )

Flucytosine ( )

Serotype:A,B,C,D ( )

ELISA for HIV ( )

Follow up:

Died=1,Discharged home=2. ( )

APPENDIX V: LABORATORY PROCEDURES

CRAG Test: Cryptococcus Antigen Latex Test is a simple qualitative or semi-quantitative, 5 minute test to detect polysaccharide antigens associated with Cryptococcus neoformans infection.

Qualitative Testing:

1.The test latex was resuspended by rapidly inverting the bottle several times and held in a vertical position. The bottle was squeezed to deliver one drop of Test Latex into each test circle.

2.Using pipettes provided in the test kit, one drop of CSF was dispensed into reaction circles.

3.With the paddle end of the pipette used to deliver the specimen, the specimen and Test Latex were mixed thoroughly and spread over the entire surface area of the reaction circle. The pipette was discarded after this step.

4.The card was placed on a clinical rotator set to rotate at a 100 rpm for five minutes.

5.The slide was tilted to obtain a flow pattern and each circle carefully examined for any agglutination.

Interpretation of the test:

-Any Test Latex clumping or clearing observed immediately after the five minute rotation step was considered a positive result.

-The absence of agglutination of the Test Latex was considered a negative result.

Positive and negative controls were carried out simultaneously.

India ink stain:

1.The CSF from container 1 was centrifuged for 5-10 mins and the supernatant fluid removed. The sediment was mixed.

2.A drop of the sediment was transferred to a slide where a drop of India ink was added, and the slide covered with a cover glass and examined under 40x objective.

3.Cryptococcus neoformans was identified as oval or round cells, irregular in size measuring 2 -10 μm and surrounded by a large unstained capsule.

CSF fungal culture:

After inoculating the routine plates, the entire amount of CSF available was centrifuged for 20 minutes at 5000 rpm. The sediment was inoculated into BHI (Brain Heart Infusion) broth and incubated at 37 degrees centigrade for 48 hours. Blind subcultures were performed every 24 hours for twenty days in saborouds media. The subcultures were incubated for upto three days before reading the growths. Crytococci sometimes take upto three weeks for growth. The above protocol needed this requirement. The broths were discarded after twenty-one days if they failed to grow any fungi.

Susceptibility testing:

Having identified the organism, anti-fungal susceptibility tests were undertaken following the National Committee on Clinical and Laboratory Standards (NCCLS) protocol. Susceptibility to Amphotericin B, Fluconazole, Miconazole Flucytosine were determined.[77]

Interpretation of MICs according to NCCLS recommendations(µg/ml)

	Ampho B	Fluconazole	Miconazole	5Flucytosine
Sensitive	<2	<8	<1	<8
Intermediate Resistant	-	8	-	8-16
Highly Resistant	>2	>16	>1	>16

Serotyping of Cryptococcus neoformans:

A cryptocheck agglutination kit was used for serotyping.

One drop of suspension of isolate is added to 20 µl of typing serum on a black porcelain card. After mixing well and rotating at 100 rpm for 5 minutes, the reaction is noted. A positive reaction is indicated when the suspension gets clumped and the respective sero type noted.

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