STUDIES ON INCIDENCE OF *Cryptococcus neoformans* IN CEREBROSPINAL FLUID OF HIV POSITIVE INDIVIDUALS IN BHAGALPUR DISTRICT OF BIHAR,

A Thesis

Submitted to

The West Bengal University of Animal and Fishery Sciences
In partial fulfilment of the requirements for the Degree of
Master of Veterinary Science

In

VETERINARY PUBLIC HEALTH

By

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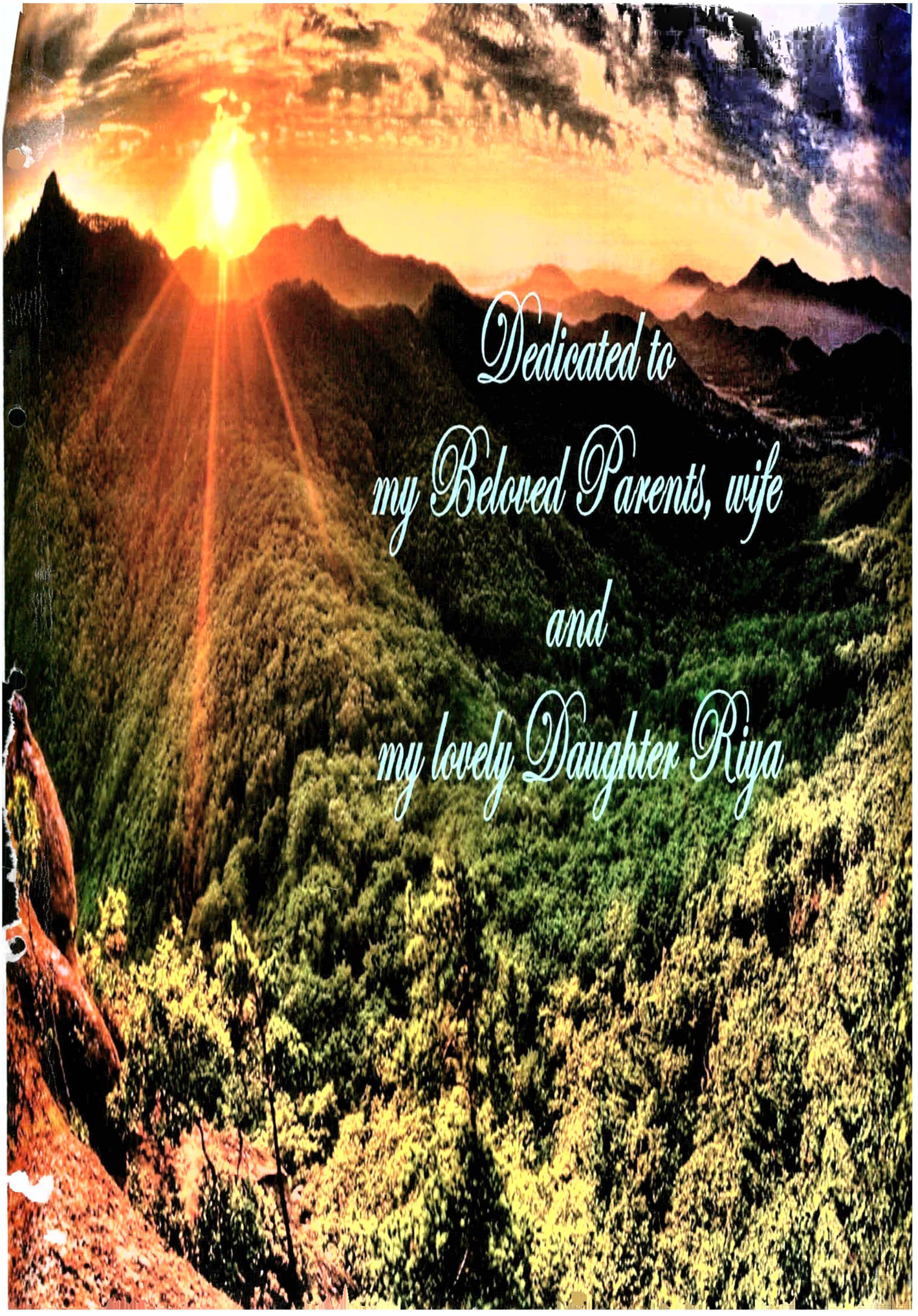
Dedicated to
my Beloved Parents, wife
and
my lovely Daughter Riya

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled "***STUDIES ON INCIDENCE OF Cryptococcus neoformans IN CEREBROSPINAL FLUID OF HIV POSITIVE INDIVIDUALS IN BHAGALPUR DISTRICT OF BIHAR", work carried out by Dr. Rakesh kumar*** in partial fulfillment of the requirements for the award of the **'Degree of Master of Veterinary Science in Veterinary Public Health, submitted to** West Bengal University of Animal and fishery Sciences, Belgachia, Kolkata-37, is the faithful and bonafide research work carried out under my personal supervision and guidance. The researches of the investigation in this thesis have not so far been submitted for any other degree or diploma.

The assistance and help received during the course of investigation have been duly acknowledged.

(Dr. Chanchal Debnath)
Chairman,
Advisory Committee

Dated: 28/10/, **2015**

Kolkata

APPROVAL OF THE EXAMINATION FOR THE AWARD OF THE

DEGREE OF MASTER OF VETERINARY SCIENCE IN

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We, the undersigned, having been satisfied with the performance of Dr. Rakesh kumar in the viva-voce examination, conducted today, The...08./.01.....2016, recommended that the thesis entitled "***STUDIES ON INCIDENCE OF Cryptococcus neoformans IN CEREBROSPINAL FLUID OF HIV POSITIVE INDIVIDUALS IN BHAGALPUR DISTRICT OF BIHAR***" be accepted for the award of the degree of Master of the Veterinary Science in Veterinary Public Health.

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Rakesh kumar

Rakesh Kumar

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ABBREVIATION

%	Percentage.
°C	Degree centigrade.
()	First bracket.
<	Less than.
>	Greater than.
≤	Less than or equal to.
≥	Greater than or equal to.
=	Equal to.
,	Coma
	Colon
;	Semi-colon.
(+)	Positive.
(-)	Negative.
/	Per
@	At the rate of
AIDS	Acquired Immuno Deficiency Syndrome.
Approx.	Approximate
ANNOVA	Analysis of variance.
CSF	Cerebro Spinal Fluid.
CPC	Capsular polysaccharide.
CNS	Central Nervous System.
CC	Cyclohexamide Chloramphenicol.
CDC	Centers for Disease Control and Prevention.

CLSI	Clinical Laboratory Standard Institute.
Cm	Centimetre.
DMSO	Di- Methyl Sulph-Oxide.
DTM	Dermatophytes Test Medium.
et al	And other.
etc.	And many others.
Fig.	Figure.
F/O	Faculty Of.
Gm	Gram.
Gr	Group.
Hrs	Hours.
H_2SO_4	Sulphuric acid.
HIV	Human Immunodeficiency Virus.
i.e.	That is.
$K_2Cr_2O_7$	Potassium dichromate.
KOH	Potassium Hydroxide.
Lit	Litre.
Lb	Pounds.
LCB	Lactophenol Cotton Blue.
L.P.	Lumber Puncture.
M	Molarity.
MICs	Minium Inhibitory Concentration.
MIC_{50}	Minium Inhibitory Concentration 50%
MIC_{90}	Minium Inhibitory Concentration 90%
µm	micro gram.
mg	Miligram(10^{-3}g).
Ml	Mililitre(10^{-3}L).

mm	Milimeter(10^{-3}m).
Mg/kg	milligram per kilogram.
mg%	Milligram percentage.
Min	Minute(s).
MOPS	3-(N-mopholino) propanesulfonic acid, monosodium salt
M.V.Sc.	Master in Veterinary Science.
N	Normality.
NCCLS	National Committee for Clinical Laboratory Standards.
No.	Number.
N.S.	Normal Saline.
OD.	Optical Density.
OMA	Oat Meal Agar.
OMCA	Oat Meal Cereal Agar.
ppm	Parts per million.
PH	Negative logarithm of hydrogen ion concentration.
PDA	Potato Dextrose Agar.
r.p.m.	Revolutions per minute.
SDA	Sabouraud Dextrose Agar.
Spp.	Species.
S/C	subcutaneous.
sec.	Seconds.
Temp.	Temperature.
USA	United States of America.
USP	United States Pharmacopeia.
UV	Ultraviolet.
Var.	Variety.
VAS	Veterinary and Animal Sciences.

VPH	Veterinary Public Health.
v/v	Volume by Volume.
v/w	Volume by weight.
Viz.	Namely.
W.B.U.A.F.S.	West Bengal University of Animal and Fishery Sciences.
w/v	Weight by volume.
WHO	World Health Organization.

CHAPTER ONE

INTRODUCTION

Cryptococcosis is a highly infectious systemic disease caused by *Cryptococcus species*. It is also known as Torulosis or European Blastomycosis or Busse-Buschke's Disease. It affects man and variety of animals and also occasionaly reported in birds, reptiles and amphibians. Normally it is an opportunistic pathogen and causes disease in an immunocompromised person whose immune system is weakened or absent and who is less capable of battling infections because of an immune response that is not properly functioning. Examples of immunocompromised people are those having HIV or AIDS, are pregnant, or are undergoing chemotherapy or radiation therapy for cancer. Other conditions, such as certain cancers and genetic disorders, can also cause a person to become immunocompromised. Immunocompromised individuals can sometimes be prone to more serious infections and/or complications than healthy people. They are also more prone to getting opportunistic infections, which are infections that do not normally afflict healthy individuals. According to WHO in conjunction with UNICEF around 175 countries including India have people living with HIV/AIDS. These people are always at risk to acquire this infection. Cryptococcosis can cause life threatening infections like meningoencephalitis. Cryptococcosis, one of the AIDS defining infections, considered as "sleeping disease" became an "awakening giant" within a couple of years and has now been predicted as the "Mycosis of the future," with a predilection that for every million patients with AIDS, 50,000–100,000 will contract Cryptococcosis (Nayak *et al.*, 2010).

Cryptococcus is a basidiomycetous yeast-like fungus which causes respiratory and neurological disease in humans and animals

(Casadevall *et al.,* 1992 and Lewis *and* Rabinovich, 1972). The encapsulated yeast is a major cause of fungal meningitis and meningoencephalitis especially in immunocompromised patients (Park *et al.,* 2009 and Bratton *et al.,* 2012). It is a saprobe in nature, with a worldwide distribution and is especially common in pigeon droppings. While birds are not affected, they apparently harbor the organism in a commensal form (Darzé *et al.,* 2000).

Presently the etiological agent of Cryptococcosis is classified under the Order *Tremellales*, Class *Tremellomycetes*, Phylum Basidiomycota and the Kingdom *Fungi* and it includes at least 37 different species in which, two are most important human pathogens namely *Cryptococcus neoformans* and *Cryptococcus gattii* (Gupta and Fries, 2010). Initially in the year 1894, Sanfelice isolated the organism from the peach juice and it was named as *Saccharomyces neoformans* (Sanfelice, 1894). Later in 1901, Vuillemin proposed a new genus *Cryptococcus* to include these yeasts, based on the differences found in morphological aspects when compared to *Saccharomyces* sp (Vuillemin, 1901). In 1975, Kwon-Chung identified teleomorph (sexual stage) of *C. neoformans* and described it as *Filobasidiella neoformans* on the basis of the result of mating of two strains of serotype D (Kwon-Chung, 1975).

C. neoformans has a worldwide distribution and has been associated with a variety of environmental sources particularly in bird excreta and decaying wood (Walter *and Coffe,* 1968; Ruiz *et al.,* 1981). It has also been isolated from heartwood of several tree species in South America (Lazera *et al.,* 2000) and India (Randhawa *et al.,* 2006). This mycosis causes life threatening infections in approximately 7-15% of patients with HIV/AIDS around the world, and up to 40% in Africa (Dzoyem *et al.,* 2012). The main habitat of the fungus is the digestive tract of the bird (mainly pigeons, parrots, and canaries). It is saprophytic and is present in soil, nests, and other sites contaminated with excreta of pigeon, parrots, and canaries. The

infection occurs either by inhalation of spores or through direct inoculation in the skin (Garg and Mahajan, 2004). The spores of *C. neoformans* var. grubii are found in *Eucalyptus camaldulensis* bark and spores of *C. neoformans* var. gattii are found in *Eucalyptus camaldulensis, Eucalyptus tereticornis, Ficus religiosa and Syzigium cumini* trees (Gugnani *et al.,* 2005) and that of *C. neoformans* var. neoformans are also found in *Ficus religiosa, Syzigium cumini* and *Tamarind indica* trees (Randhawa *et al.,* 2006).

C. neoformans is an opportunistic pathogen that mainly affects immune-compromised persons (Tintelnot *et al.,* 2004) while, *C. neoformans* var. gattii has an aggressive behaviour and affects mainly immune-competent individuals (Kwon-Chung *et al.,* 1984 and Frasés *et al.,* 2009). The serious impact on human health can be attributed to the number of virulence factors present in *C. gattii* and *C. neoformans* which share structural and physiological characteristics that allow them to invade and survive in host tissues (Lupo *et al.,* 2008). The major virulence factors linked with Cryptococcus are the ability to grow at 37°C (Nichols *et al.,* 2007), the production of the pigment melanin (Kwon-Chung *et al.,* 1992 and Panepinto *et al.,* 2009) and the formation of a polysaccharide capsule (Zaragoza *et al.,* 2009).

On the basis of capsular antigen, there are four serotypes namely A, B, C, and D. Serotype A is present in *C. neoformans* var. grubii, Serotype D is present in *C. neoformans* var. neoformans and Serotype B and C is present in *C. neoformans* var. gattii. Serotype AD consists of hybrids between A and D varieties. On the basis of molecular characteristics *C. neoformans* var. neoformans contains type VNIV, *C. neoformans* var. grubii contains type VNI and VNII, while hybrids between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* are type VNIII. *C. gattii* contains the molecular types VGI, VGII, VGIII and VGIV. This molecular basis of typing is

mainly used in epidemiological studies of outbreaks as per the guidelines provided by Centres for Disease Control and Prevention (CDC).

C. neoformans grows as a budding yeast in vitro with typical cell size ranging from 5-10 µm in diameter (Okagaki *et al.,* 2010) and its spore measures 1-2 µm in diameter (Giles *et al.,* 2009 and Velagapudi *et al.,* 2009). Cryptococcus is a yeast that can reproduce both sexually and asexually. *C. neoformans* is highly susceptible to 70% ethanol, 0.5% chlorhexidine, 1.2% sodium hypochlorite, iodophors (e.g., betadine), phenolic disinfectants, glutaraldehyde and formaldehyde. *C. neoformans* can also be killed by moist heat of 121°C for a minimum of 20 minutes or dry heat of 165-170°C for 2 hours (CDC).

In humans it causes infections ranging from asymptomatic colonization of airways to respiratory signs or disseminated infections which may involve Central Nervous System (CNS), eyes, skin, and other organs (prostate gland, bone, adrenal glands *etc*). In lungs, pleural effusions occur. Patients may feel shortness of breath or dyspnoea, pleuric chest pain or hemoptysis. Other signs may include low-grade fever, weight loss, anorexia and malaise. In immunocompromised patients, it causes serious respiratory syndromes and progressive pulmonary disease. In brain, cranial nerve paralysis is common. In immunocompetent patients, cryptococcomas may cause focal signs such as aphasia, cerebellar syndrome or paresis. Elevated cerebrospinal fluid (CSF) pressure from cryptococcomas or chronic meningoencephalitis can lead to hydrocephalus and further neurological signs, including dementia. Other syndromes such as spinal cord lesions or ischemic stroke also occur (Centre for Food Security and Public Health).

The administration of highly active antiretroviral therapy (HAART) has resulted in a decrease in the number of cases of AIDS-related cryptococcosis in developed countries, but *Cryptococcus* is still a major

problem in developing country where HAART is not readily available (Perfect *et al.*, 2010).

India is one of the major HIV/AIDS burdened countries. The incidence of Cryptococcosis, one of the important opportunistic pathogens in HIV/AIDS patients in India, particularly in Bihar State have not yet been well-studied and therefore an epidemiological survey of these infection among these patients has been undertaken in the present study. Study of the zoonotic potentiality of the pathogen is also an important aspect has not yet been well explored. Therefore, the present study was chalked out keeping in mind the following objectives:

1) Isolation and identification of *Cryptococcus spp* present in HIV/AIDS positive patients.

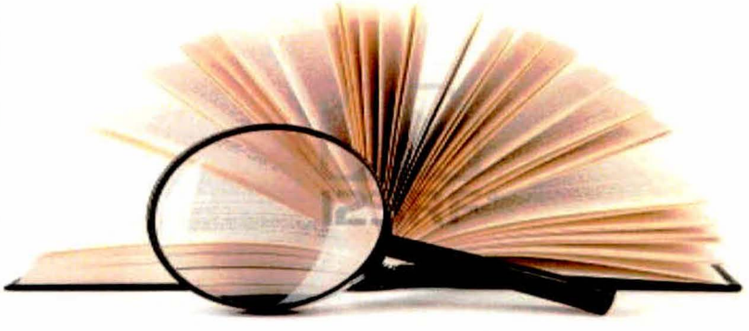
2) Molecular study of the isolates on the basis of Randomly Amplified Polymorphic DNA (RAPD) analysis.

3) Epidemiological study of the disease among the population considered.

4) Study of the zoonotic potentiality of the pathogen.

5) Determination of the antifungal susceptibility pattern of the isolates.

CHAPTER TWO



Literature Review

REVIEW OF LITERATURE

2. A. HISTORICAL BACKGROUND

In 1894, pathologist Busse and surgeon Abraham Buschke first described this yeast as a human pathogen when they isolated a `*Saccharomyces-like*' organism from a bone infection in a young woman (Busse, 1894) later that year Francesco Sanfelice reported the isolation from fermenting peach juice of a similar yeast, which he termed *Saccharomyces neoformans* because of its unique colony form (Sanfelice, 1894). In 1905, the yeast was identified as a CNS pathogen when von Hansemann described the first case of cryptococcal meningitis. The genus Cryptococcus has been recognized in fruit juice, milk, and soil and pigeon droppings for more than 125 years (Casadevall A and Perfect JR, 1998) *C. gatti* specifically was first noted as a new strain when found in a CSF sample from a Congolese boy in the 1960 (Vanbreuseghem, Takashio,1970 and Sorrell., 2001).

First case of Cryptococcosis in cat and dog was reported by Curtis in 1951 (Curtis, (1951), Holzworth, (1952), Holzworth and Koffin, (1953), McGrath, 1954, Barron, (1955), Trautwein and Nielsen, (1962), Johnston and Lavers, 1963.) and by Seibold in 1953 (Seibold *et al.,* 1953) respectively. Human Cryptococcosis was first reported from Spain in 2003. It was isolated from a 60 years old heterosexual Spanish farmer when he visited the hospital since he was suffering from cephalalgia and somnolence (Francisco *et al.,* 2005) with increasing frequency cryptococcus infections are being reported in United States (US). The first case of cryptococcosis caused by *C. neoformans var. grubii* in a new species of bandicoot (*Bandicota indica*) is described. The animal was trapped in a bamboo thicket in a park located in the city of Jabalpur, India (Singh *et al.,* 2007) after 2009, there have been reports of this infection increasing tremendously (Shawn *et al.,* 2013).

2. B. TAXONOMY

The Cryptococcus is classified under Kingdom Fungi, Phylum Basidiomycota, Class Tremellomycetes, Order Tremellales, and Family Tremellaceae. *C. neoformans* and *C. gatti* are basidiomycetes, encapsulated yeast. *C. neoformans* and *C. gatti* can be sub-classified into four serotypes and two species with two varieties. Their serotypes are based upon capsular agglutination reaction and are designated A, B, C, or D. Under the species neoformans, Serotype A and D cryptococci were previously classified. However, it has been proposed that serotype A cryptococci be considered as a separate variety based upon genotypic differences (Franzot *et al.,* 1999) B and C serotypes are exclusive to what have become, a species distinct from *Cryptococcus neoformans* (Kwon-chung *et al.,* 2002 and Sharpton *et al.,* 2008) recognition of rare hybrids of *Cryptooccus gattii* and *Cryptococcus neoformans* provide evidence of taxonomic proximity, but not identity, between the two species (Bovers *et al.,* 2008).

Recently, molecular studies using different methods, such as PCR fingerprinting, Amplified Fragment Length Polymorphisms [AFLP] analysis, Restriction Fragment Length Polymorphism [RFLP] and Multilocus Sequence Typing [MLST], have shown that *C. neoformans* and *C. gattii* can also be grouped in at least eight cryptic species defined as molecular types, therefore characterizing intraspecies and inter-species genetic diversity (Ngamskulrungroj *et al.,* 2009,) thus, although other subgroups exist, the main genotypes recognized so far are: *C. neoformans* var. *grubii*, serotype A, molecular types VNI=AFLP1 and VNII=AFLP1A; the hybrid serotype AD corresponds to VNIII=AFLP3 and *C. neoformans* var. *neoformans*, serotype D, which consists of VNIV=AFLP2. In both serotypes B or C of *C. gattii,* the molecular types established are VGI=AFLP4, VGII=AFLP6, VGIII=AFLP5, and VGIV=AFLP7 (Meyer *et al.,* 2009) the pathogenic implications of these molecular types will require more studies, since the epidemiological distribution is not completely known.

However, it has recently been shown that the genotype VGIIa presents higher virulence than VGIIb isolates of *C. gattii* (Ngamskulrungroj *et al.,* 2011).

2. C. GENUS DESCRIPTION

Cryptococcus (hidden sphere) is a genus of fungus. In culture these fungi grow as yeasts. The capsule of *Cryptococcus neoformans* is highly rich in glucoronic acid and mannose, having O-acetyl groups. (Ross and Taylor 1981) this is the main virulence factor in cryptococcal infections (Casadevall and Perfect, 1998) also it is believed that virulence factor is due to melanin which is produced when *C. neoformans* is cultured on Niger or Bird-seed agar (Labrecque *et al.,* 2005) *C. gattii* contains heavy and polysaccharide capsule and has ability to grow at human body temperature causing it to be a pesky human pathogen, allowing its resistance to antifungal medications (Galanis *et al.,* 2009).

Cryptococcus albidus can be differentiated from *C. neoformans* by giving phenol oxidase test negative and shows cream colour when grown on Niger or bird-seed agar (Labrecque *et al.,* 2005) *C. albidus var. albidus* is a unique in terms of both temperature range (grows in between 25°C and 35°C) by violating Van Uden's rule which states that yeast strains of a particular species cannot have their maximum growth temperature vary by more than 5°C and its ability to assimilate lactose but not galactose another variety *C. albidus var. diffluens* can also be distinguished from *C. neoformans* by assimilating melibiose but not galactose (Labrecque *et al.,* 2005).

2. D. SPECIES DESCRIPTION

Infectious species	Non-infectious species
C. neoformans	*C. adeliensis*
C. grubbi	*C. aerius*
C. gattii	*C. albidosimilis*
C. laurentii	*C. antarcticus*
C. albidus	*C. aquaticus*
C. uniguttulatus	*C. ater*
	C. bhutanensis
	C. consortionis
	C. curvatus
	C. phenolicus
	C. skinneri
	C. terreus
	C. vishniacci

2. E. MORPHOLOGY

Mercedes *et al.,* (1967) studied the morphology of *Cryptococcus neoformans* through electron microscope. They described the ultra thin sections and capsule containing microfibrils (30 to 40 Å in diameter) that appeared to radiate from the cell-wall which coiled and intertwined in various directions. *Cryptococcus neoformans var. gattii* shows the unusual presence of elongated cigar shaped morphology in cerebrospinal fluid in a Congolese Bantu boy (Vanbreuseghem, Takashio , 1970 and Sorrell, 2001).

Schell (2006) described *C. neoformans* exist as a yeast form in the environment .

Shea (2007), Ryan (2004) and Dale (2007) described *Cryptococcus neoformans* as a spherical yeast (4 to 6 µm in diameter) that produces a capsule containing glucoronoxylomannam (GXM), extending the overall diameter to 25 µm or more.

Shea (2007) stated that *C. neoformans* usually has a single bud that pinched off at the mature stage.

Karkowska *et al.*, (2009) described the *C. neoformans* can be differentiated into several complicated morphological forms, including yeast, clamydiospores, pseudohyphae and hyphae, and is typically present in yeast form during infection. Small-sized basidiospores (1.8 to 3.0 µm) can turn into yeast cells, the form preffered at 37°C or can form dikaryotic hyphae which are favoured as 24°C.

2. F. ISOLATION OF CRYPTOCOCCUS FROM ENVIORNMENT

Yamamoto *et al.*, (1995a ,1995b) and Denton and Di Salvo, (1968) and Ajello (1958) and stated that their is high prevalence of *C. neoformans* in pigeon excreta. He isolated the *C. neoformans* from both pigeon and chicken habitats.

Walter and Yee (1968) described the growth inhibitory effect of chicken droppings on *C. neoformans* to the presence of a high molecular growth inhibitory substance and the high alkalinity of the droppings, which may explain the failure of isolation of the organism from chicken excreta.

Ruiz *et al.*, (1981) studied the occurrence of the agent of cryptococcosis in the areas of this study which could be due to the environmental conditions favouring growth of *C. neoformans* such as a large amount of pigeon excreta, dry excrement, and a suitable pH. Other studies have previously reported a more frequent isolation of the yeast from dry rather than from moist excrement. Dry excrement is a favourable substratum since it has fewer bacteria and thus less competition, which could easily explain the higher population density found in this substratum.

Lazera *et al.*, (1993) showed that the percentage of positive samples of *C. neoformans* var. *neoformans* in pigeon droppings from

heavily contaminated *downtown* locations (26·3%), is similar to available data for other Brazilian cities and other countries.

Yamamoto *et al.*, (1995a) studied strain typing analysis by random amplified polymorphic DNA (RAPD) to confirm the genetic correlation between environmental isolates of *C. neoformans* and clinical isolates from patients with pulmonary cryptococcosis.

Emmons (1995) studied a frequent saprobic association of *C. neoformans* with the old excreta and nests of pigeons.

Yamamoto *et al.*, (1995) reported the isolation of *C. neoformans* from pigeon excreta in Nagasaki in which 4 samples out of 8 samples were positive (50%).

Kielstein (1996) showed that the increase of pH is not regarded as responsible for the survival of *C. neoformans* in the birds droppings.

Pal (1997) isolated the prevalence of *C. neoformans* from pigeon droppings which was 7 of 28 samples (25%) in Kathmandu.

Montenegro and Paula (2000) collected 38 samples from large heaps of pigeon droppings in the city of Sao Paulo, Brazil out of which 10 (26·3%) were positive for *C. neoformans*. They also collected samples from Twelve eucalyptus woods and vegetable material from *Eucalyptus* spp. Trees and *C. neoformans* var. *gattii was isolated from Eucalyptus* wood in Ibirapuera Park. Also *Eucalyptus cameldulensis was isolated from Eucalyptus* wood in Ibirapuera Park and Aclimacao Park in Sao Paulo, Brazil.

Sorvillo *et al.*, (1997) and Bogaerts *et al.*, (1999) and Bell *et al.*, (2001) observed that there is seasonal variation in isolation of *C. neoformans* from patients with AIDS and is more during rainy season.

Chee and Kim (2003) isolated *Cryptococcus neoformans* and investigated it in pigeon droppings collected from only three localities in Seoul.

Misuzu *et al.*, (2004) isolated *Cryptococcus neoformans* from chicken faeces in suburban areas of Thailand from 36/150 houses

(24.0%) in the dry season and 6/150 houses (4.0%) in the rainy season and all environmental isolates were of serotype A. Kuroki et al., 2004 isolated the C. neoformans from chicken faeces in suburban areas of Thailand which was 24.0% .

Kwang and Soo (2005) collected twenty nine samples of pigeon droppings (n = 12) and soil contaminated with avian excreta (n = 19), collected from different sites in Busan, were examined for isolation and characterization of Cryptococcus neoformans and of these samples, 5 strains of *C. neoformans* were recovered from pigeon droppings (5/12 : 41.7%).

Rosario and Colom (2008) stated that pigeons were not the only reservoir of *Cryptococcus* and other birds could act as reservoir in Spain.

Lugarini *et al.*, (2008) isolated *C.neoformans* from faeces of parrots and sparrows and stated that faeces of domestic birds and those around as act as reservoir for *C.neoformans.*

Majid *et al.*, (2010) isolated samples of pigeon droppings in urban environmental sources at places with a large population of Ahwaz, Iran out of the 65 samples about 22 samples (34%) were positive for *C. neoformans.*

Doracild *et al.*, (2013) evaluated 122 samples of dried pigeon excreta collected in 49 locations in the City of Cuiabá, State of Mato Grosso, Brazil, including public squares (n = 5), churches (n = 4), educational institutions (n = 3), health units (n = 8), open areas covered with asbestos (n = 4), residences (n = 23), factory (n = 1) and a prison (n = 1).

Xavier *et al.*, (2013) collected Thirty three samples of pigeon droppings from 10 different regions in Tiruchirappalli district out of which thirty three samples, 20 samples (60.6%) were positive forC. *neoformans*

2.G. EPIDEMIOLOGY

Francoise *et al.*, (1985–1993) studied out of the 1,057 cases of cryptococcosis (1,013 patients), 827 (86%) involved patients with AIDS and the increasing number of cases of human immunodeficiency virus (HIV)-related cryptococcosis over time paralleled the AIDS epidemic except for a higher male-to-female ratio. They found out that Malignancies (32%), organ transplantation (19%), and corticosteroid therapy (33%) were the main predisposing conditions in 163 patients without AIDS.

Sara A Mirza *et al.,* (1992–2000) conducted survey during 1992–2000 in 2 areas of the United States (Atlanta, Georgia, and Houston, Texas, metropolitan areas; combined population, 7.4 million) and a total of 1491 incident cases were detected, of which 1322 (89%) occurred in HIV-infected persons. They also reported that the annual incidence of cryptococcosis per 1000 persons with AIDS decreased significantly during this study period, from 66 in 1992 to 7 in 2000 in the Atlanta area, and from 24 in 1993 to 2 in 2000 in the Houston area. They also stated that Poisson regression analysis revealed that African American persons with AIDS were more likely than white persons with AIDS to develop disease.

Dromer *et al.,* (2004) described that the total number of cryptococcosis cases evolved were in parallel to that recorded for HIV-infected patients and the changes occurring after HAART introduction were analysed and they found out that a negative binomial regression model established about 46% decrease of the incidence of cryptococcosis during the post-HAART era (1997–2001, n = 292) compared to the pre-HAART era (1985–1996, n = 1352).

Schop (2007) observed the *Cryptococcus neoformans* serotype A appears to be implicated in 99% of AIDS patients with Cryptococcosis worldwide, except in France where serotype A is responsible for 80% of the infection. It is also estimated that 6% to 10% of patients with AIDS in United States, Western Europe and Australia and 0% to 50% of AIDS

patients in sub-saharan Africa are infected with life threatning cryptococcal meningitis .

Lin (2009) studied the differences in number of cases in number of strains of *C. neoformans* serotype A and D are distributed world-wide and cause the vast majority of cryptococcal infections, predominantly in immunocompromised individuals. Serotype A is responsible for over 95% of Cryptococcosis cases worldwide. More frequent cases of serotype A and D had been reported in Europe where Cryptococcosis is associated with 77% of HIV patients. *Cryptococcus gattii* strain B and C are localised in tropical and sub-tropical regions (e.g. Australia, Papua New Guinea and South America) and cause cryptococcal infections mostly in immunocompetent hosts .

Lin (2009) studied the isolates of *C. gattii* cause the infections in regions with temperate climate. Cryptococcal meningitis, caused by the fungus *C. neoformans*, can cause a upto 30% mortality in AIDS patients in resource-poor regions such as South east Asia. By the 1990s, *C.neoformans* had become the leading cause of culture-positive meningitis in many regions, including New York City. Cryptococcal meningitis alone kills 624,000 people each year.

Park *et al.,* (2009) studied the incidence of *C. neoformans* which ranged from 0.04 to 12% per year among HIV persons. They found out that Sub-Saharan Africa had the highest yearly burden estimate (median incidence 3.2%, 720 000 cases; range, 144 000–1.3 million) and Median incidence was lowest in Western and Central Europe and Oceania (≤0.1% each). Further they estimated approximately 957 900 cases (range, 371 700–1 544 000) of cryptococcal meningitis occur each year, globally resulting in 624700 deaths (range, 125 000–1 124 900) by 3 months after infection.

2. H. GROWTH AND PHYSIOLOGY

Miller *et al.,* (1990) stated that the growth rate of *C. neoformans* depends on a variety of conditions. The exponential doubling

time among different strains of Cryptococcus varies from 2.5 to 6 hour in media at 37°C.

Bruatto *et al.,* (1992) reported that C. *neoformans* has minimal growth requirements of simple carbon and nitrogen sources, and even vitamin supplementation with thiamine may not be required for growth.

Zimmer and Roberts (1979); Li and Wu (1992) observed that most strains readily excrete large amounts of urease in the presence of urea, and its detection in vitro has led to a rapid urease test for the identification of *C. neoformans.*

Jacobson and Petro (1987) stated that since *C. neoformans* does not produce hydroxamate siderophores, but responds to iron in the environment, it probably has specific surface receptors for the acquisition of iron.

The unique biochemical feature of *C. neoformans* is its ability to produce diphenol oxidases, which may function as antioxidants and enhance survival of the yeast in the host was reported by Tinnell (1993). and Jacobson *et al.,* (1994).

Perfect *et al.,* (1993) described that when the purine pathway gene for phosphoaminoimidazole carboxylase (*ADE2*) is inactive, *C. neoformans* cannot grow and produce disease in an immunocompromised host.

Two external physiological conditions have been linked to growth and regulation of the capsule in *C. neoformans.* Both the pCO2 concentration and ferric iron availability dramatically influence growth and capsular size (Granger, *et al.,* 1985 and Vartivarian, *et al.,* 1993) recent studies indicated that *C. neoformans* cells transcribe, process, and secrete pheromones in response to starvation (Moore and Edman, 1993).

Martinez *et al.,* (2001) stated that *C. neoformans* and *C. gattii* are the only cryptococcal species to grow at 37°C.

2. I. VIRULENCE FACTORS

Virulence is mainly due to following factors,

1) **CAPSULAR POLYSACCHARIDE (CPS)** :-

Kozel and Cazin Jr (1971) and Fromtling *et al.,* (1982) stated that the capsule of *C. neoformans* is clearly a virulence factor. In their experiment on cryptococcosis, they found that capsule-free isolates or mutants are less virulent than encapsulated wild-type cells.

In some other studies conducted by Farmer and Komorowski (1973); Levinson *et al.,* (1974) and Milchgrub *et al.,* (1990) it was observed that human cases due to capsule-free or small capsule isolates have a stronger host response, greater inflammation, and less severe disease.

Dykstra *et al.,* (1977) stated that Virulence is not correlated with capsule size. Strains of *C. neoformans* vary in virulence for animals, but virulence is not correlated with the amount of capsular polysaccharide (CPS).

Kwon-Chung *et al.*, (1982) and Rhodes *et al.,* (1982) Studied the pathogenicity requires the production of phenol oxidase, growth at 37°C, and the presence of a capsule.

Bulmer and Sans (1968); Kozel and Mastronianni (1976) described the capsule or CPS potentiates infection, depresses inflammation, inhibits phagocytosis and suppresses both cellular and humoral immunity (Breen, *et al.,* 1982).

Ankenbauer and Nester (1990), Mo and Gross. (1991) observed in many circumstances, this response permits the invading microorganism to grow or survive in a hostile environment. These mechanisms may be activated by specific molecules, such as phenolic compounds or sugars, as has been shown for phytopathogens.

2) Phenol oxidase and melanin production:-

Kwon-Chung *et al.,* (1982) and Rhodes *et al.,* (1982) described the Genetic analyses support the association of virulence with phenol oxidase production.

Polacheck *et al.,* (1990) observed and associates have shown that a mutant lacking phenol oxidase was killed by the epinephrine oxidative system in the presence of a transition metal ion and hydrogen peroxide. The wild type was resistant, which suggests that phenol oxidase may consume epinephrine and protect *C. neoformans* from this oxidative system in the CNS.

Polak (1990) and Jacobson (1993) observed the end product of the activity of phenol oxidase, melanin can function as an antioxidant, which may protect *C. neoformans* from oxidative host defences.

Jacobson *et al.,* (1994) observed The utilization by phenol oxidase of natural catecholamines (e.g norepinephrine and dopamine) as substrates for melaninogenesis may somehow relate to the unexplained neurotropism of *C. neoformans*.

Other determinants:-

Brueske (1986) studied the secreted proteinases which may contribute to the breakdown of host tissue or humoral proteins have been described In addition to the capsule and phenol oxidase. other features of *C. neoformans* may contribute to its virulence.

Kwon-Chung et al., (1992) observed an undefined virulence attributes is apparently linked to the mating type locus.

GalXM and mannoprotein.

Cherniak and Sundstrom (1994) described the Culture filtrates of *C. neoformans* also yield smaller amounts of another polysaccharide, GalXM, as well as a mannoprotein, which appears to be this immunodominant antigen that is responsible for evoking CM.

2. J. CRYPTOCOCCUS PATIENTS ASSOCIATED WITH AIDS

Kovacs *et al.*, (1985) and Zuger *et al* (1986) stated that Cryptococcosis is a leading mycological cause of morbidity and mortality among AIDS patients.

Powderly (1993) observed that, in most of the cases suffering from Cryptococcosis is the first indication of AIDS and incidence of life-threatening cryptococcal infections among patients with AIDS has been estimated at 6 to 10% in the United States, western Europe, and Australia and 15 to 30% in sub-Saharan Africa.

Clark *et al.*, (1990) and Powderly (1993) have reported the incidence of cryptococcus highly in the southeastern United States and equatorial Africa and this period of initial therapy, 10 to 25% of these patients die, and 30 to 60% succumb within 12 months .

2.K. INTERACTION BETWEEN C. NEOFORMANS AND HIV

Ikeda *et al.*, (1982) and Bottone *et al.*, (1987) isolated *C. neoformans* from AIDS patients that has been examined had serotype A, as compared to 75% of isolates from patients without AIDS.

Kovacs *et al.*,(1985) and Chuck and Sande (1989) studied that the first manifestation of HIV infection is cryptococcus in 26 to 45% of patients.

Orendi *et al.*, 1993 and Pettoello-Mantovani *et al.*,(1993) an observed in recent survey indicate that *C. neoformans* appears to potentiate HIV infection.

Bottone, *et al.*, (1986) reported that isolates of *C. neoformans* from AIDS patients were small-capsuled, although the patients had high circulating titres of capsular antigen. This unconfirmed observation supports the theory that isolates of *C. neoformans* from patients with AIDS differ from other isolates. These researchers discovered that the AIDS isolates developed larger capsules in mice, but comparisons of virulence or other properties were not performed .

2.L. DIAGNOSIS

Dixit *et al.*, (2009) studied the cryptococcal diagnosis is based on environmental exposure history, coupled with appropriate clinical symptoms or radiological findings, and ideally, histopathological evidence of tissue invasion. Cryptococcosis is usually diagnosed by detecting the organism or its antigens in blood, or in tissues and fluids from affected sites (e.g., cerebrospinal fluid, bronchial washings, urine (Centres for food security and public health).

Diagnosis is made on following points,

1) DIRECT MICROSCOPIC EXAMINATION

Lazera *et al.*, (1993) described the Laboratory investigation of cerebrospinal fluid (CSF) which is traditionally based on microscopic examination of India ink preparations and the direct examination was first made through India ink diluted at 1:5. Hageage and Harrington (1984) collected aspirates and similar specimens from HIV patients and effectively treated with a solution of calcofluor white and examined under a fluorescent microscope and studied the cryptococcal organisms.

Perfect (2010) described the Dark field microscopy or India ink by mixing of biologic fluids to identify the 5 to 10 μ-diameter encapsulated yeasts remains a rapid and effective method for diagnosing cryptococcal meningitis. Gomori's methenamine silver (GMS) fungal stain differentiates the narrow-based budding yeast in tissue as compared to Gram stain which usually reveals a poorly stained gram-positive yeast.

2) CULTURE METHODS

Lacaz *et al.*, (1998). cultured the cerebrospinal fluid (CSF) on Sabouraud's glucose agar and incubated at 37 degree celcius for five or eight days and saw the creamy, white mucoid colonies of cryptococcus.

Yagupsky and Menegus (1990) observed that in one analysis of this method, two blood cultures were sufficiently sensitive (70%) to detect cryptococcemia in patients with AIDS.

Staib (1962) and Chaskes and Tyndall (1978) and Kwon-Chung and Bennett (1992) tolobserved that Phenol oxidase leads to the formation of melanin, which can be demonstrated by the acquisition of a brown to black pigment when *C. neoformans* is grown on Staib's birdseed agar or caffeic acid medium.

Ellis and Pfeiffer (1990) and St.-Germain and Beauchesne (1991)., Warren and Shadomy (1991) or by a DNA-based method (Hoy *et al.*, 1989 and Mitchell *et al.*, 1994) isolated can be readily identified to the species level by several commercial systems on the basis of biochemical reactions.

Denning *et al.*, (1990) described the primary isolation medium that contains a substrate for phenol oxidase to facilitate the recognition of *C. neoformans* among other microorganisms in nonsterile specimens, such as sputum and urine. Kwon-Chung *et al.*, (1982) observed that at the subspecies level, the two varieties of *C. neoformans* are differentiated by the color reaction when grown on canavanine- glycine-bromthymol blue agar.

3) SEROLOGY

Goodman *et al.*, (1971) described the serological tests for the detection of cryptococcocal polysaccharide antigen in serum and CSF which are extremely accurate for the diagnosis of invasive disease and both latex agglutination and enzyme immunoassay tests are more than 90% sensitive and specific.

Snow and Dismukee (1975) stated that With the help of proper treatment of specimens boiling and pronasem2-mercaptoethnol treatment, false positive tests are not common when CSF titers are 1:4 or higher.

2.M. BIOCHEMICAL CHARACTERISTICS OF CRYPTOCOCCUS SPECIES

CHARACTERISTICS	Cryptococcus neoformans var. neoformans	Cryptococcus neoformans var. gattii
Teleomorph	*Filobasidiella neoformans* var. *neoformans*	*Filobasidiella neoformans* var. *bacillispora*
Phenol-oxidase production	Yes	Yes
Malate assimilation	No	Yes
Feedback repression of creatinine deaminase	Yes	No
Canavanine susceptibility	Yes	No
Glycine assimilation (% of strain)	10 to 20	100

(Reference:- Kwon-Chung, K. J., and J. E. Bennett. 1992, Littman, M. L., and R. Borok. 1968)

2.N. PCR

Thomas *et al.*, (1994) observed the unique oligonucieotides and designed for the amplification of specific DNA for *C.neoformans*.The amplification of 37 strains of *C.neoformans* DNA by the using combination of primers CN4 and CN5. And also used only for C.neoformans other pairs of primers like CN5-CN6,CN4-ITS1,andCN6-ITS1.

Yoshihiro *et al.*, (1995) isolated the *C.neoformans var. neoformans* (serotype A) from Southern Japanese prefecture of Nagasaki and using random amplified polymorphic DNA profiles through the three primers revealed six patterns from 21 clinical and three pattern from 8 isolates in which pattern first (18 of 29 isolates) is the most common

throughought the entire Nagasaki Prefecture. But patterns fourth are exclusively isolates from Nagasaki City.

Kwang and Soo (2005) isolates of *C.neoformans* and studied the genetic variability through the random amplified polymorphic DNA (RAPD) by using three 10-mer primers in which two different RAPD patterns clearly distinguished the identified of isolates .

Tay *et al.*,(2005) isolated the 20 strains *C.neoformans* serotype A out of 544 samples of bird excreta from Klang Valley, Malaysia and using the pheromone-specefic PCR assay to describe the all are of alpha- mating type.

Seddi *et al.*, (2010) observed the ID32C auxanogram panel (BioMerieux, Marcy 1'Etoile,France) for the identification of Cryptococcus neoformans complex and species is identified by the multiplex PCR using the primers of CN70 and CN49 ,and described the mating type of PCR.

2.O. PATHOGENECITY/TOXICITY

Rozenbaum and Goncalves (1994) Observed the diseases which include meningio-encephalitis (77.2%), pulmonary Cryptococcosis mostly in immunocompromised host, 8.2%), and several disease . Disseminated cryptococcosis is a complication and may occur in 91.8% of cases.

Day (2004) stated that *C. neoformans* causes various disease in immunocompromised and immunocompetent host.

Baronetti *et al.*, (2006) described the *C. neoformans* can cause systemic infection, including fatal meningitis (meningio encephalitis) in normal, diabetic, and immunocompromised hosts

Schop (2007) and Lin (2009) studied the serotypes A and D are opportunistic pathogens while serotypes B and C may infect immunocompetent individuals.

Lin and Doering(2009) observed the Cryptococcosis may be fatal if untreated, Spores or dessicated yeasts cells of *C.neoformans* enters the host respiratory tract by inhalation.

Doering (2009) described the pulmonary infection disseminates most commonly to the brain and the skin.

Karkowska-Kuleta *et al.*, (2009) described the infection from *C. neoformans* in the brain can be fatal if untreated.

CNS infection:

Bicanic and Harrison (2005) stuied the If cryptococcal meningitis occurs,than the mortality rate is between 10-30%.

Day (2004) and Dale (2007) described the cryptococcosis of the CNS presents mostly in the form of acute, subacute and chronic meningitis, with symptoms of persistent headache, nausea, dizziness, ataxia, impaired memory and judgment, irritability, somnolence, clumsiness, and confusion . As the disease progresses, seizures may occur. CNS infection may also present as a brain abscess (cryptococcomas), subdural effusion, dementia, isolated cranial nerve lesion, spinal cord lesion, and ischemic stroke.

Respiratory infection:

Day (2004) it also studied the respiratory system infections include pneumonia, cavitation, enobronchial masses, empyema, nodules, sinusitis, mediastinitis, bronchiolitis obliterans, and pneumothorax.

Dale et al., (2007) observed the pulmonary cryptococcosis may present as cough, dyspnea, blood-streaked sputum, and a dull ache in the chest.

Cutaneous infection:

Day (2004) it also again studied in cutaneous infection include lymphadenitis, pancreatitis, hepatitis, peritonitis, osteomyelitis, septic arthritis, myositis, endophthalmitis, papilloedema, optic nerve atrophy, pyelonephritis, prostatitis, endocarditis, fungaemia, myocarditis, Cushing's syndrome, adrenal mass lesions, thyroiditis.

Dale et al., (2007) it also studied in skin lesions may be single or multiple and commonly begin as painless lesions of the face or scalp. Skin lesion may take the form of erythematous or umblicated papules,

Corpe and Parr (1953) and Kahn *et al.*, (1985) observed that the pulmonary cryptococcosis in the immunocompetent host has occurred as a coinfection with both *Mycobacterium tuberculosis* and echinococci (Dalgleish, 1981).

Lambertus *et al.*, (1990) described that pulmonary cryptococcosis may follow as a consequence of steroid therapy for *P. carinii* pneumonia.

Perla *et al.*, (1985) and Murray *et al.*, (1988) reported that the adult patients may suffer from C *.neoformans* then show adult respiratory distress syndrome.

2) CNS:-

Crump et al., (1992) described that the Cranial nerve palsies and papilledema are the most common ocular manifestations seen in patients with cryptococcal CNS invasion.

Rozenbaum and Goncalves, (1994) stated that Cryptococcemia often precedes CNS invasion and may persist for an extended period of time (1-16 weeks) despite treatment.

Mitchell and Perfect (1995) described that infection typically presents as a subacute process characterized by headache, fever, and, less often, altered mental status however, characteristic of either acute or chronic meningitis can occur.

McGuireD *et al.*, (1997) described the complications of CNS infection which include hydrocephalus, motor or sensory deficits, cerebellar dysfunction, seizures, and dementia.

Perfect (2010) observed that the symptoms may not be typical, and patients may present with acute(several days) symptoms of severe headaches, with intermittent headaches, or even no headache but with altered mental status.

4) SKIN

Cawley *et al.*, (1950) and Littman and Zimmerman. (1956) and Sarosi *et al.*, (1971) and Schupbach and Wheeler and Briggaman

(1976) described that *C. neoformans* has been shown to cause almost every type of skin lesion.

Gauder (1977) observed that the Cutaneous involvement often occurs concurrently with cryptococcosis in the brain or other organs.

Concus *et al.*, (1988) described that AIDS patients may have umbilicated papules that resemble molluscum contagiosum.

Casadevall *et al.*, (1994) stated that Skin lesions may present as acneform lesions, purpura, papules, vesicles, nodules, tumors, abscesses, ulcers, superficial granulomas, plaques resembling ecchymosis, and sinus tracts. Dissemination of organisms to the skin can cause a variety of lesions, which may mimic other diseases. Papules, which may ulcerate or evolve to other forms, are often seen initially.

5) PROSTATE

Braman(1981) observed that the prostate gland has been long recognized as a site for cryptococcal localization .

Plunkett *et al.*, (1981) studied that the infection does not usually cause symptoms of prostatitis, but the yeasts have been isolated from prostatic tissue and blood after urological procedures.

Perfect and Seaworth (1985) and Blocker *et al.*, (1987) observed the a vulvar lesion andue to the infection of *C. neoformans* and also caused a penile ulcer.

6) EYE

Beyt (Jr.) and Waltman. (1978) observed that the eye may also be a portal of entry for *C. neoformans.* There is a case report of the transmission of Cryptococcosis from the donor of a corneal transplant to a recipient, who later developed meningitis.

Doft and Curtin (1982) which can also be simultaneously infected with other pathogens, such as HIV and cytomegalovirus.

Perry and Donnenfeld (1990) described a case of cryptococcal keratitis following keratoplasty.

Denning *et al.*, (1991) described that most cases of cryptococcal endophthalmitis lead to severe visual loss, and only an occasional case is successfully managed.

Johnston *et al.*, (1992) and Rex *et al.*, (1993) described that catastrophic loss of vision in patients without evidence of endophthalmitis.

Crump *et al.*, (1992) described the manifestations range from ocular palsies to involvement of the retina.

Pema et al., (1994) observed that ocular signs and symptoms in approximately 45% of all patients with meningitis.

2.Q. ZOONOSES AND PUBLIC HEALTH SIGNIFICANCE

Lagrau *et al.*, (2005) stated that case of cryptococcal meningitis in an immunocompetent female patient with exposure to a pet magpie (Pica pica) and genetically indistinguishable isolates were cultured from the cerebrospinal fluid of the patient and excreta of the bird. Their data strongly suggested zoonotic transmission of *C. neoformans var. grubii* from a magpie to this patient.

Shrestha *et al.*, (2004) reported a case of cryptococcal pneumonia in a patient who was taking infliximab for rheumatoid arthritis and this exposure history raised the possibility that the patient acquired the infection from his pet cockatiel. So they advised the patients receiving infliximab to avoid exposure to pet avian excreta.

Micalizzi *et al.*, (1997) reported the case of a pigeon keeper who presented with a post traumatic lesion of his third right finger. Cryptoccoci were found in the lesion in the absence of other underlying disorder. Treatment with oral itraconazole achived clinical reolution of the lesion., primary cutaneous Cryptococcosis is a rare event. It may be occur in immunocompromised hosts, but its development in the immunocompetent patients without any sign of systemic disease is exceptional.

Nosanchuk *et al.*, (2000) stated that the patient's infection resulted from exposure to aerosolized cockatoo excreta. They further advised the immunocompromised patients to avoid pet birds and avian excreta.

2.R. ANTIFUNGAL RESISTANCE

Casadevall *et al.*, (1993) studied an increase in the fluconazole MICs for serial *C. neoformans* isolates that were recovered from five patients with recurrent cryptococcal meningitis.

Paugam *et al.*, (1994) reported clinical and *In vitro* fluconazole resistance in three AIDS patients with recurrent cryptococcal meningitis (increases in MICs from 4 to 64, 16 to 128, and 0.25 to 16 mg/ml, respectively).

Franzot and Hamdan (1996) and Pfaller *et al.*, (1998) showed that the resistance to antifungal drugs is rare among clinical isolates of *C. neoformans* but has been reported. The use of antifungal agents, particularly in long-term suppressive regimens, has raised concern about the development of drug resistance in *C. neoformans*.

Klepser and Pfaller (1998) described an extensive survey of the susceptibility profiles of clinical isolates of *C. neoformans* at a university hospital during 1987 to 1994 which helped to allay these fears by indicating no emergence of resistance.

Pfaller *et al.*, (1999) studied the similarity between the MICs of *C. neoformans* isolates from Malawi and the United States which concurs with data from a previous study of 164 African and 402 North American clinical isolates of *C. neoformans* which were tested and found to be susceptible to fluconazole and other triazoles, with over 99% inhibited by concentrations of fluconazole <32 µg/mL.

Pfaller *et al.*, (1999) and Chandenier *et al.* (2004) and Aller *et al.*, (2005) and Brandt *et al.*, (2005) described that Voriconazole and posaconazole have consistently good activity against *C. neoformans* .

Datta *et al.*, (2003) demonstrated that the intermediate susceptibility to fluconazole in 16% of clinical isolates which is the first

report from India on higher fluconazole MIC values in clinical cryptococcal isolates.

Scorzoni et al., (2007) described that the Ethanol extracts (EELLsal) was the most active among the crude extracts, with MICs of 62.5 lg/ml for *C. neoformans.* The Ethanol extract (EELLsal) presented moderate activities against the Candida strains used and strong activity against C. neoformans. This Ethanol extract (EESLsal) which presented MICs of 250 lg/ml for Candida strains and 125 lg/ml for *C. neoformans* was considered moderately active against all yeasts tested.

Perfect *et al.*, (2010) observed that the use of voriconazole and posaconazole is still restricted to salvage settings, as no clinical trials have been undertaken to compare these agents to first-line drugs and these agents remain prohibitively expensive for use in resource-limited settings. Funari *et al.*, (2011) explained the strongest activity with an MIC of 25.0 lmol/l (or 15.6 lg/ml) against *C. neoformans*, which was approximately 6 times less active than the positive control amphotericin B (MIC of 4.3 lmol/l).

Funari et al., (2011) stated that Ethyl acetate and n-butanol fractions from the ethanol extract of L. Salviaefolia leaves (FAc2 and FBu2, respectively) showed strong inhibition of fungal growth, along with verbascoside and asebogenin previously isolated from L. salviaefolia. Therefore, these natural products might be considered promising prototypes for the development of new antifungal agents, especially against *C. neoformans.*

Warnock *et al.*, (1998) and Petrou *et al.*, (2000) and Maxwell *et al.*, (2003) and Dannaoui *et al.*, (2006) and Dias *et al.* (2006) described that Cryptococcus MIC values to the azoles which have been generated by Etest are higher than those generated by broth micro-dilution method.

Chandenier *et al.*, (2004) and Pfaller *et al.*, (1999) and Aller *et al.*, (2007) observed that isolates of *C. neoformans* remained highly susceptible to 5-flucytosine (100% susceptible at MIC of 4 μg/ml), fluconazole (100% susceptible at MIC of 4ug/ml) as well as to other azoles

like voriconazole & itraconazole. Voriconazole exhibited the highest inhibitory activity (GM 0.056 mg per ml) followed by itraconazole with GM of 0.069 mg per ml. Voriconazole and other newer azoles like posaconazole have consistently been shown to have good activity against *Cryptococcus neoformans*.

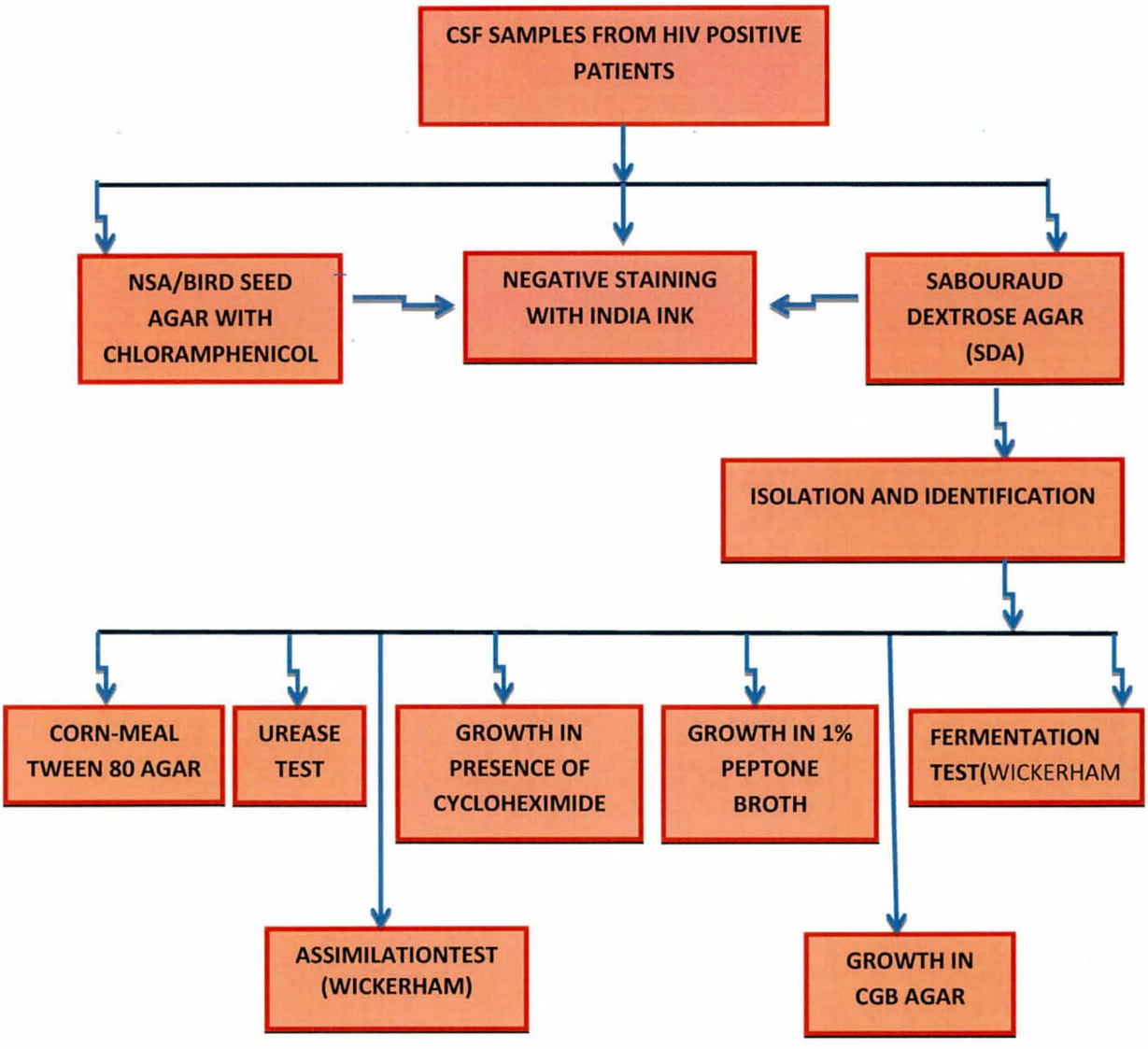
Chowdhary *et al.*, (2011) reported the fourfold increase in fluconazole MICs over a period of 1.5–2.5 months in 4 serial isolates obtained from four patients receiving antifungal therapy of amphotericin B for 3 weeks followed by fluconazole prophylaxis (400 mg daily) from Delhi involving both clinical and environmental isolates.

Rajani *et al.*, (2014) studied that the Antifungal susceptibility testing of the *isolates* was done by CLSI M27 A-3 methodology for amphotericin B, fluconazole, voriconazole, itraconazole and 5-flucytosine in which drugs no resistance develop among clinical isolates of *Cryptococcus neoformans*, but only two isolates showed slightly higher minimum inhibitory concentration (MIC) to 5-flucytosine (8 μg/ml).

CHAPTER THREE

MATERIAL AND METHODS

The methodology adopted in the present work was described by Manoharan *et al.*, (2001)



Two states namely Bihar and Jharkhand have shown an increase in number of HIV patients in recent years which is against the National trend of decrease in number of HIV patients (Source: The Telegraph, Calcutta, Monday, December8, 2014). This observation leads to select Eastern Bihar as the area for the present work.

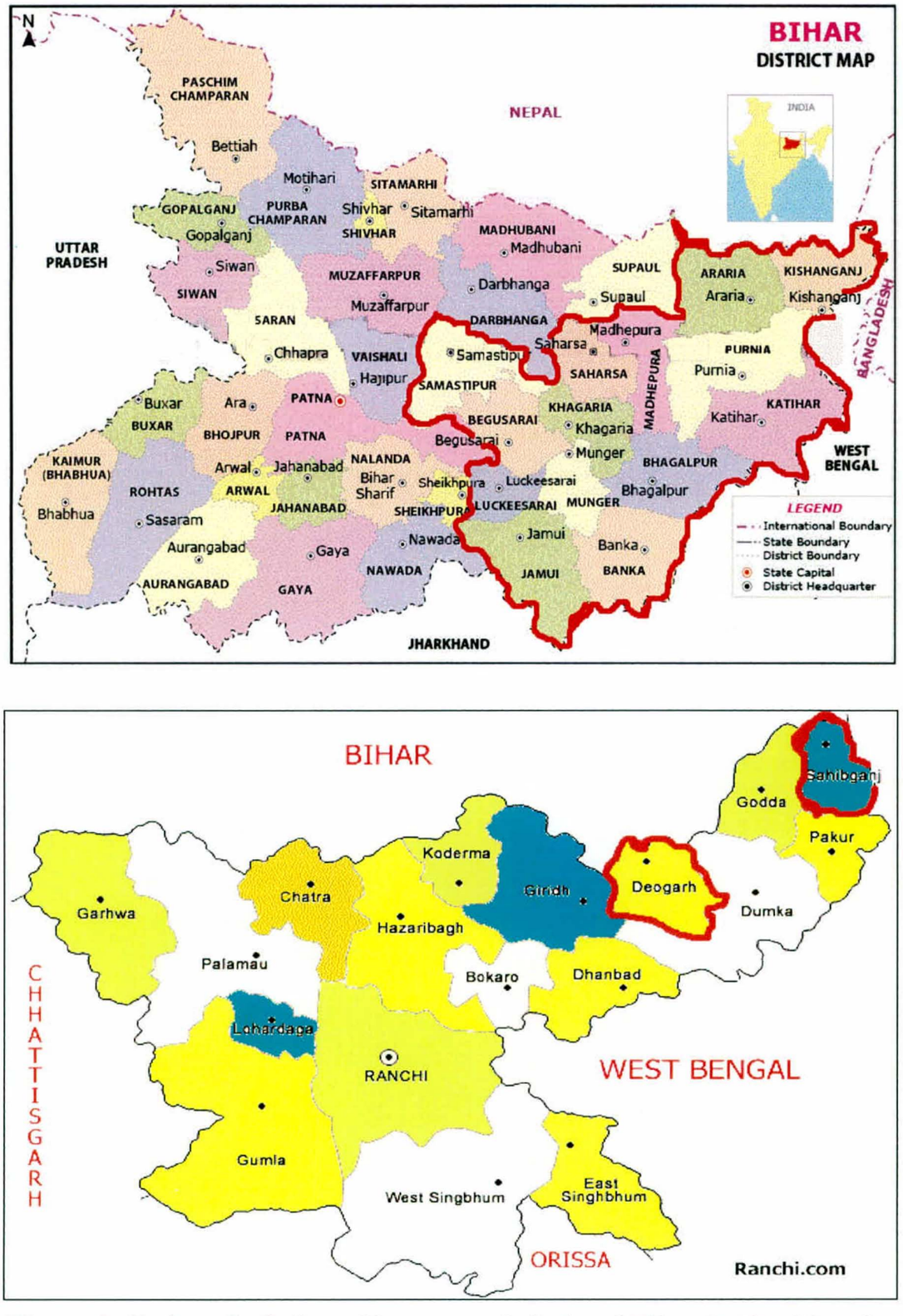


Figure 1: Red encircled are the areas of study of Bihar & Jharkhand.

The Jawaharlal Nehru Medical College and Hospital, Bhagalpur, Bihar was identified as one of the important anti-retroviral treatment (ART) centres of the Eastern Bihar covering 14 districts (Bhagalpur, Banka, Katihar, Kisanganj, Purnea, Araria, Saharsa, Madhepura, Khagaria, Begusarai, Samastipur, Lakhisarai, Munger, Jamui) of Bihar and 2 districts from Jharkhand (Deoghar, Sahebgunj).

MATERIALS:

GLASS WARE:

High quality Borocil® glassware,resistant to heat and chemicals were used for this study. The petri dishes.test tubes,Durham tubes(6×50mm),screw cap bottles, flasks, beakers, measuring cylinders etc. were cleaned and dipped over night on mixture of sulphuric acid and potassium dichromate(equal parts of H_2SO_4 and K2Cr2O7, v/w), washed thoroughly with distilled water,rinsed several times in sterilized distilled water and air dried. The petri dishes were wrapped in clean craft paper. The hollow-glass goods were plugged with cotton wool and sterilized in hot air oven at 180^{0}C for half an hour. The sterilized glass wares were stored in a clean, dust free chamber for further use.

Glass wares and plastic wares used:

TABLE 1: Glass wares and plastic wares

Sl no.	Glass wares(Borosil®)	Plastic wares
1.	Conical flasks-100ml,200ml,500ml,1lt	Pipette-10,20,25ml
2.	Test tubes(various sizees)	Centrifuge tubes(Tarsons®)-1.5 ml,2ml,15ml, and 50 ml
3.	Bootles-15ml,100ml,200ml,400ml,500ml	Thermo tubes(Thermo scientific®)-0.2 ml, 0.5 ml
4.	Beakers-100ml,250ml,500ml	Bulb
5.	Measuring cylinders-250ml,500ml	Micro-tips(Axygen scientific®))-10 µl,100 µl,1 ml, 5 ml
6.	Durham tubes(6×50mm)	Aspirator(L shaped)
7.	Slides	Cover slip
8.	Petri plates(Tarsons®)	PCR tubes 0.2 ml
9.	Loop	

CHEMICALS AND REAGENTS:

Analytical grade chemicals manufactured by Himedia®,India; Merck®, Germany and Sigma®, USA, claimed to be free from the traces of noxious metal and other contaminating molecules that can interfere with growth of fungi were used in this study, Sulphuric acid, Ethanol (25 mg/disk) etc. Carbohydrates disks (Lactose, Glucose, Trehalose, Glactose, Maltose, Fructose, Sucrose, Manitol).

TRIPLE DISTILLED WATER:

Sterile triple distilled water was prepared in the laboratory by using the Borosil® Triple distillation plant in the department of VPH, F/O-VAS, WBUAFS, KOLKOTA,W.B, INDIA.

INSTRUMENTS:

The instruments like forceps, scalpels, scissors, spatula, curettes etc. Used for collection of samples were washed, cleaned, dried and wrapped in hard paper and sterilized in hot air oven for 30 minutes at 180°C or autoclaved at 121°C at 15 lbs pressure for 15 minutes.

CULTURE MEDIA:

Dehydrated culture media, manufactured by the Himedia®, India and Difco BBL®, USA were used in this study. The media were re-hydrated, sterilized and dispensed in sterilized petri dishes/ culture tubes, as required, strictly in accordance with the instruction of the manufactures. The following media were used for routine work in the present study (Annexure-C):

1. Bird's seed agar(NSA) with Chloramphenicol.
2. Sabouraud dextrose agar(SDA) with Chloramphenicol and Cyclohexamide(SDA with CC supplement).
3. CGB(Canavanine Glycien-Bromothymol Blue).
4. Urease test media.
5. 1% Peotone Broth Agar.
6. Fermentation Test.
7. Assimilation Test (yeast nitrogen base/YNB).
8. Caffeic Acid-Ferric Citrate media.
9. Corn meal tween 80 agar.
10. Yeast Nitrogen Base.

Materials For Antifungal Testing

(AGAR DIFFUSION ASSAY):

Materials required:

Ezy™ MIC Strips of antifungal agents- Himedia®

Agar plates with appropriate media.

Sterile saline (0.85% NaCl) for inoculums preparation.

Sterile loops, swabs, test tubes, pipettes, forceps, applicator, template and scissors.

McFarland 0.5 and 1 turbidity standards.

Incubator (35°C).

Medium:

RPMI 1640 broth with L glutamine and 2.0 grams per liter Glucose and 0.165M per liter MOPS buffer without sodium carbonate.

Other Instruments used:

TABLE 2: INSTRUMENTS

Sl. No.	Instrument	Make
1	BOD incubator(25°C)	OVFU®(scientific & surgical pvt.Ltd.)
2	Compound fluorescence microscope	ZEISS
3	Tarsons Digital Spinot(heater & spin)	OVFU®(scientific & surgical pvt.Ltd.)
4	Incubator(35°C)	OVFU®(scientific & surgical pvt.Ltd.)
5	Weighing Balance	Eagle
6	Electronic balance	Precisa
7	UV trans-illuminator	Wealtec®
8	Laminar air flow	Klenzo flo®
9	Hot water bath	Thermo Scientific®

Sl. No.	Instrument	Make
10	Hot air oven	OVFU®(scientific & surgical pvt.Ltd.)
11	Automatic vertical autoclave	OVFU®(scientific & surgical pvt.Ltd.)
12	Freeze	Whirlpool
13	Turbidimeter	Tarson®
14	Thermal Cycler Machine	Techni
15	Micro centrifuge machine	Eppendorf
16	Gel Documentation machine	
17	Micropepettes	Borosil
18	Vaccum pump	Tarson®

METHODS:

Sample collection:

With the permission of the hospital authority a total of **40 CSF samples** from HIV positive patients were collected under the supervision of a medical expert. Samples were collected from patients suffering from secondary pulmonary or nervous breakdown type symptoms. Samples were collected in 1.5ml sterile vials by lumber puncture method. After collection samples were immediately taken to the laboratory for further examinations.

Data collection:

Relevant data of the patients like name, age, sex, stage of HIV, treatment history, locality, economic status, history of contact with household animals or birds (cattle, buffalo, parrots, pigeon, poultry, duck) *etc* were documented in a data sheet for further study.

Isolation and identification:

Direct microscopy: Small portions of the collected CSF samples were negative stained with India ink for the presence of encapsulated yeast cells of *Cryptococcus sp.*

Culturing: CSF samples were directly inoculated on two different agar media namely Sabouraud Dextrose agar (BBL®, Difco®) and Bird Seed Agar (Himedia®). Both the plates were incubated at 28°C and watched for 7-10 days for the growth of creamy white mucoid colonies on SDA plates or brown coloured colonies on Bird Seed agar plates.

Microscopy: Colonies from SDA and Bird seed agar were microscopically examined after negative staining with India ink for the presence of encapsulated budding yeast cells.

Sub-culturing: After the growth on primary culture media plates sub-culturing was done on Corn meal-tween 80 agar plates with an incubation period of 2-3 days at 28°C and observed for the presence of off-white mucoid colonies.

Biochemical tests:

a) Ability to grow at 37°C on Sabouraud medium.

b) Urease activity.

c) Carbohydrate assimilation and fermentation tests.

d) Growth on CGB (Canavanine-Glycine-Bromothymol Blue) agar media to differentiate *C.neoformans* and *C. gatti*

RAPD analysis:

For DNA extraction, chromosomal DNA of all the isolates was extracted using a commercial kit for yeast DNA extraction (Genei®, Merck®) according to the step by step instruction of the manufacturer. PCR was carried out with a 25µl reaction volume containing 12.5 µl of 2x Red Dye PCR master mix (Genei®, Merck®), 2 µl isolated DNA, 2 µl (each) RAPD primer. The following 3 primers were used for RAPD analysis: OPH-20 (5'-

GGGAGACATC-3'), OPH-02 (5'-TCGGACGTGA-3') and R-28 (5'-ATGGATCCGC-3'). These were prepared on the basis of the reports of Yamamoto *et al.,* (1995). Amplification was performed for 30 cycles in a Techni® Gradient thermal cycler with the following protocol: 4 minutes of initial heat at 94°C, 45 seconds of denaturation at 92°C, 60 seconds of annealing at 34°C, and 90 seconds of primer extension at 72°C, followed by a final extension cycle for 10 min at 72°C. The amplification products were analyzed by electrophoresis on 1.5% agarose gels in 1× TAE (Tris-acetate-EDTA) buffer at 90 V for 75 min and then stained with SyBr Green safe dye (Thermo-Fisher®). Finally the gel was observed in a gel documentation system.

ANTIFUNGAL SENSITIVITY TESTING:

Agar Diffusion Assay was adopted to determine the MIC values of Fluconazole, Ketoconazole, Itraconazole, Voriconazole, & Amphotericin-B by using MIC strips (Antifungal Ezy™ MIC Strips, Himedia®).

PROCEDURE for Agar Diffusion Test:

Materials required

Ezy™ MIC Strips of antifungal agents-Himedia®
Agar plates with appropriate media
Sterile saline (0.85% NaCl) for inoculum preparation.
Sterile loops, swabs, test tubes, pipettes, forceps, applicator, template and scissors.
McFarland 0.5 and 1 turbidity standards.
Incubator (35°C).

Medium

RPMI 1640 broth + MOPS + 2% Glucose + 1.5% agar

Media preparation for 1 litre

RPMI 1640 (contains 0.165M MOPS and L-glutamine)	46.19 g
Bacto agar	15 g
Glucose	20 g

1. RPMI powder was dissolved in 500 ml deionized water.
2. Filter sterilized with a 0.2 mm filter.
3. Glucose and Bacto agar was dissolved in 500 ml deionized water. Autoclaved for 15 minutes at 15 psi pressure (approx. 121°C) and then cooled to approx. 50°C.
4. The sterile RPMI + MOPS solution was warmed gently to approximately 45°C and mixed with the cooled glucose-agar solution. pH adjusted to 7.0.
5. The solution was then poured into sterile petridishes and allowed to solidify.

Inoculum preparation

Well isolated colonies of *C. neoformans* isolates from a 48-72 hour Sabouraud Dextrose agar plate was taken and Homogenized in 0.85% NaCl to achieve 1 McFarland turbidity.

Inoculation

A sterile, non-toxic swab (not too tightly spun) was dipped into the inoculum suspension and the swab was pressed against the inside of the tube. The entire agar surface was swabbed evenly, rotating 90° in three directions. Excess moisture was allowed to be absorbed for about 10-15 minutes so that the agar surface is completely dry before applying the Ezy™ MIC Strips.

Application

Using sterile forceps, the strips were applied to the inoculated agar surface, ensuring that the MIC scale is facing upwards, i.e. towards the opening of the plate, and that the concentration maximum is nearest the rim of the plate. The whole length of the strip was in complete contact with the agar surface.

Incubation

The agar plates were incubated at 35°C for 48 to 72 hours.

INTERPRETATION OF RESULTS

Reading the MIC

After the required period of incubation whereby growth becomes distinctly visible, the MIC value was read at the point of intersection between the zone edge and the Ezy™ MIC Strips if the end point is clear. When different growth inhibition patterns are seen, we have used the illustrations in the Ezy™ MIC Strips antifungal reading guide to correctly select the MIC end point (figures 1 and 2).

When growth occurs along the entire strip i.e. no inhibition ellipse is seen, the MIC is reported as > than the highest value on the MIC scale. When the inhibition ellipse is below the strip i.e. the zone edge does not intersect the strip, the MIC should be reported as < than the lowest value on the MIC scale.

Important reading observations

1. For amphotericin B, the end point is read at the point of complete inhibition (100%).
2. For azoles, we read the MIC at the first point of significant inhibition or marked decrease in growth density. We have used the principle of 80% inhibition to visually select the end point.

Amphotericin B (read at complete inhibition of all growth).

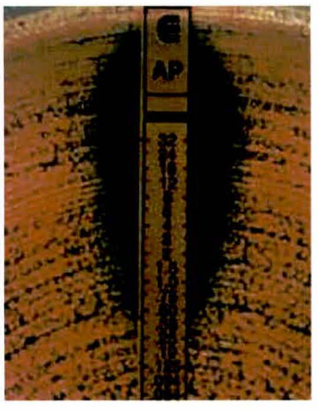


Figure 1a. MIC 1 mg/m (include all microcolonies)

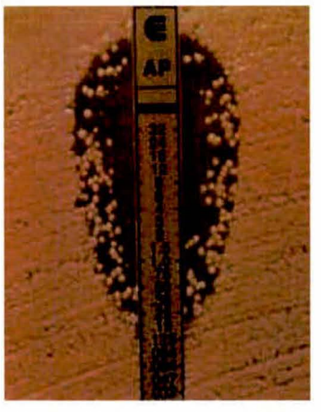


Figure 1b. MIC 3 mg/ml (macrocolonies in ellipse)

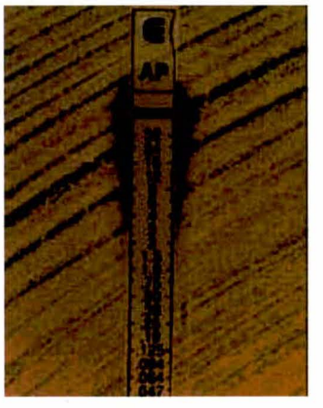


Figure 1c. MIC 4 mg/ml (small and slim ellipse)

Azoles (read at first point of significant inhibition i.e. so-called 80% inhibition).

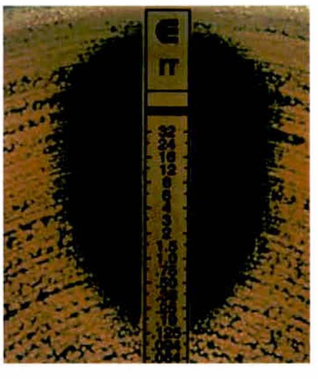


Figure 2a. MIC 0.125 mg/ml (sharp end point).

Figure 2b. MIC 0.5 mg/ml (lawn of microcolonies within a discernable ellipse).

Figure 2c. MIC >256 mg/ml (macrocolonies in ellipse).

Figure 2: Test Reading Guide for Yeasts.

TABLE 3: NCCLS M27-A document provides the following interpretive guidelines:

Antifungal	Susceptible	S-Dose Dependent	Intermediate	Resistant
Fluconazole	≤8	16-32	-	≥64
Itraconazole	≤0.125	0.25-0.5	-	≥ 1
Ketoconazole	< 0.125	-	0.05	>0.5
Voriconazole	< 1.0	2	-	>4
Amphotericin B	≤0.5	-	-	≥2

Susceptible-Dose Dependent (S-DD) - dependent on achieving maximum blood level. For fluconazole, doses of ≥400 mg/day dose may be required in adults with normal renal function and body habitus. For itraconazole, steps to assure adequate drug absorption and plasma levels of >0.5 mg/ml may be required for optimal response.

The susceptibility of these isolates is uncertain and available data do not permit them to be clearly categorized as susceptible or resistant.

STATISTICAL ANALYSIS:

Statistical analysis was carried out by applying Chi square test as described by Snedecor and Cochran(1994).

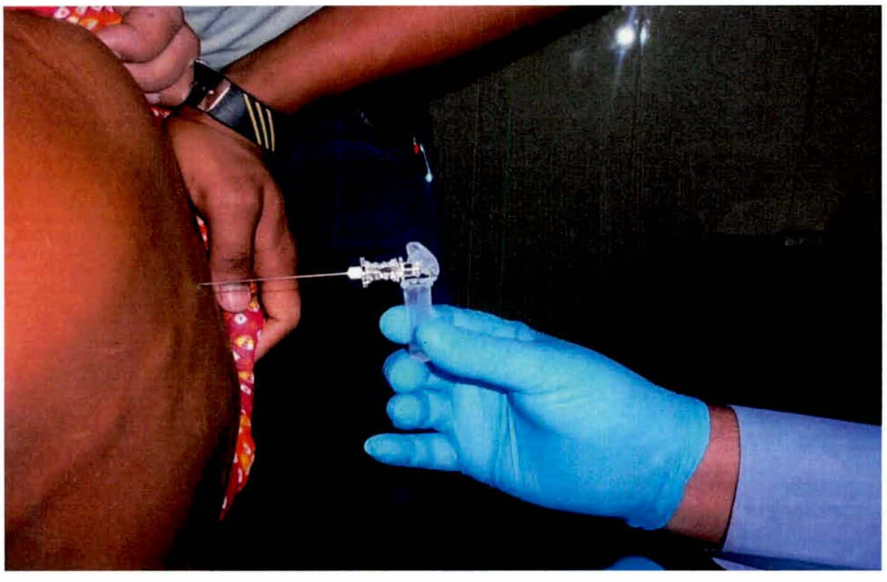


Fig 3: CSF collection by lumbar puncture

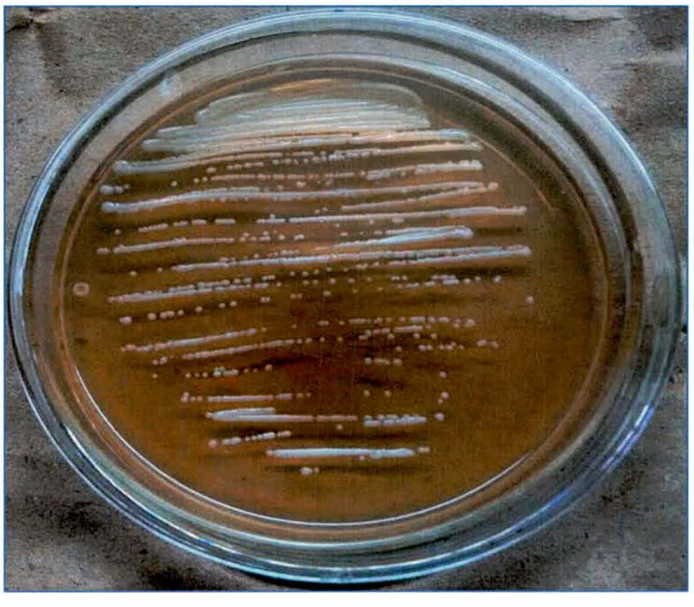


Fig 4: Showing creamy white mucoid colonies of *C. neoformans* on Sabourad dextrose agar

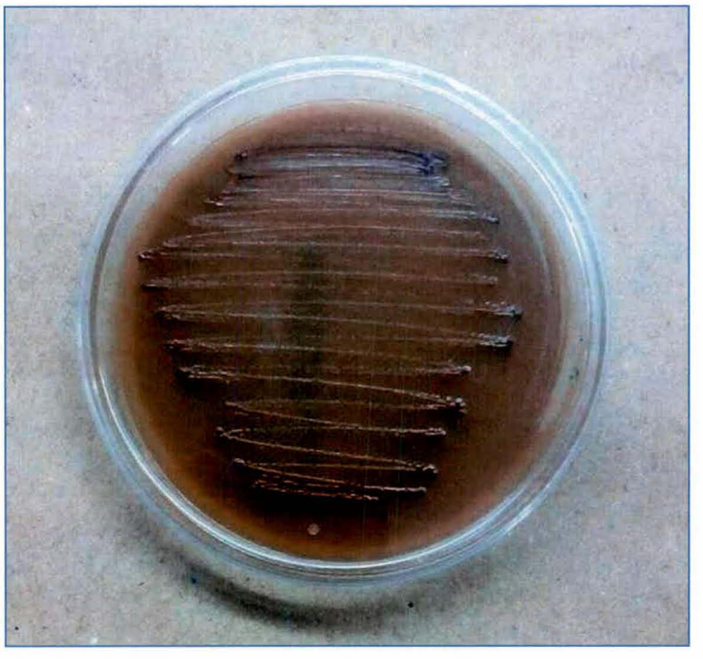


Fig 5: Showing brown coloured colonies of *C. neoformans* on Bird Seed agar

Fig 6: Growth on CGB agar. Showing colonies of *C. neoformans* with no colour change of the media (right side) and uninoculated control (right side)

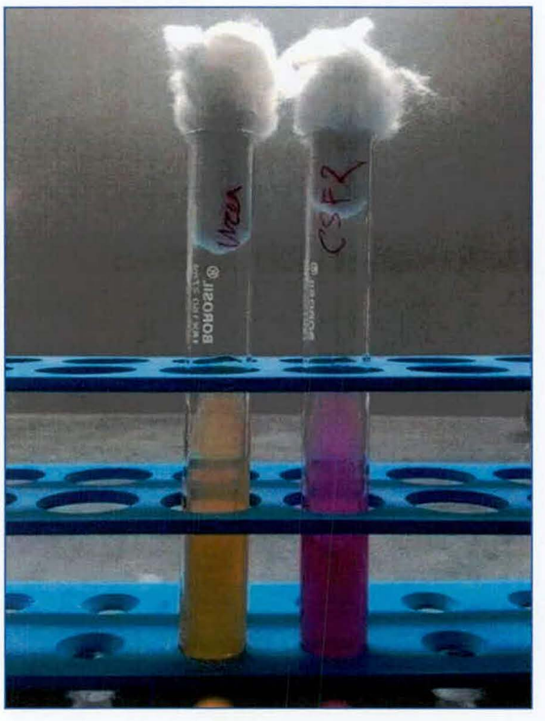


Fig 7: Urease positive activity of *C. neoformans* (right side tube). Uninoculated control (left side tube)

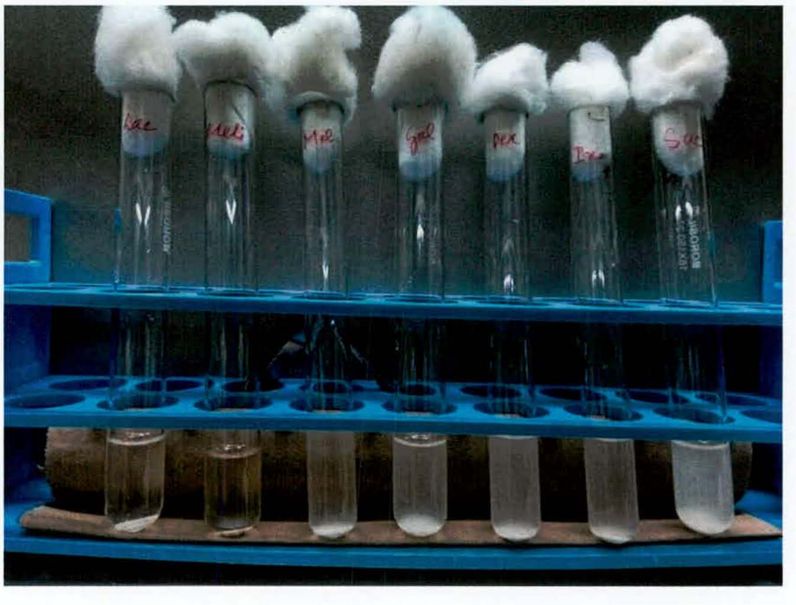


Fig8: Carbohydrate assimilation pattern of *C. neoformans*. First and second tube from left side showing no assimilation and other tubes showing positive assimilation of different carbohydrates.

Fig 9: Fermentation of different carbohydrates by *C. neoformans*. Production of no gas in the durham's tubes indicates no fermentation.

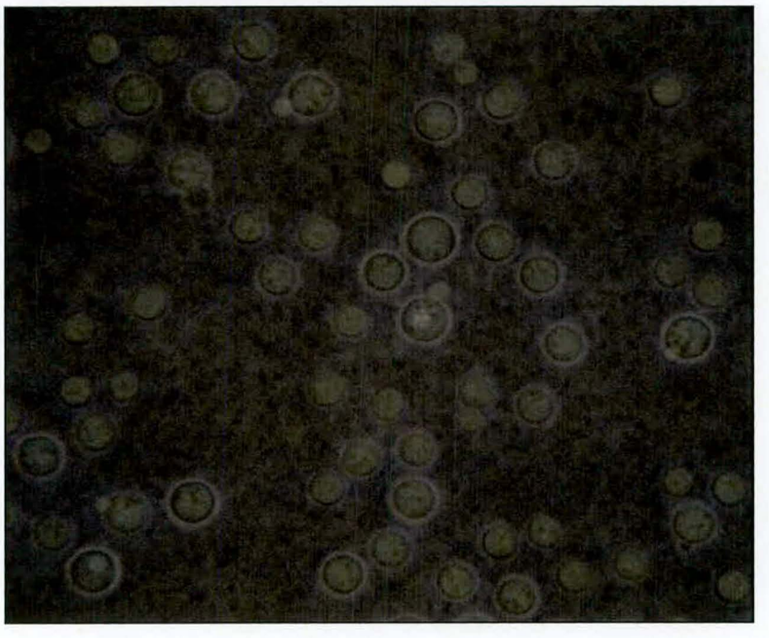


Fig 10: negative staining of C. neoformans with India ink showing circular thinly encapsulated yeast cells (under 100x phase contrast microscope)

CHAPTER FOUR

RESULTS AND DISCUSSION

The present study was conducted in a period of 8 months ranging from January through August, 2015 with the samples collected from Jawaharlal Nehru Medical College and Hospital, Bhagalpur, Bihar. In this study, a total of 14 (35.00%) numbers of *Cryptococcus neoformans* complex were isolated from 40 samples of cerebrospinal fluid (CSF) collected from HIV positive patients (table 4). The occurrence of the disease among the HIV-AIDS positive patients have also been reported by many researchers from various parts of the globe *e.g.* Schop (2004); Lin (2009); Sara *et al.*, (1992–2000); Park *et al.*, (2009); Francoise *et al.*, (1985–1993) and Dromer *et al.*, (2004). The findings of the present study is in agreement with the findings of Schop (2007), who reported the occurrence of the disease in HIV-AIDS patients from Sub-Saharan Africa ranging from 0% to 50%. The wide variation in his findings may be due to the coverage of a wide geographical area which is at a level of a sub-continent, whereas the present study is from a part of a state of India. Otherwise, the finding of the present study (35% positivity in HIV-AIDS patients) is in between of the occurrence reported by him.

Of the total 40 samples, characteristic spherical encapsulated yeast cells was observed for 14(35.00%) samples under microscope after negative staining by India ink.

All those 14 samples produced typical brown-coloured colonies upon culturing on Bird seed agar cultures. In **1992, Kwon-Chung and Bennett** documented that *C. neoformans* and *C. gattii* are the only yeasts of this kind capable of synthesizing melanin through the conversion of hydroxyl benzoic substrates by phenol oxidase activity in bird seed agar (Staib agar),which showed a coffee-brown coloration. Colonies suspected to be Cryptococcus produced a brownish colour on BSA (Fig No:-5), after being transferred to Sabourad Dextrose Agar media at both temperatures of 25^{0}C and 37^{0}C, gave cream-coloured, smooth, mucoid yeast like colonies (Fig No:-4). On evaluation, encapsulated yeast cells were seen with Indian Ink

(Fig No:-10). Urease test was also positive and was indicated by change of colour from yellow to pink red (Fig No:-7).

All the isolates were found to be positive for characteristic carbohydrate assimilation (Fig No:-8) and fermentation profile (Fig No:-9). Assimilation was indicated by the presence of turbidity in the YNB medium upon agitation, showing the reliability for isolation of *C. neoformans*. Production of gas in the beef extract broth into the Durham tubes were recorded as positive fermentation.

The positive isolates which were confirmed to be *C. neoformans* were further tested on Canavanine-Glycine-Bromothymol blue (CGB) agar for the differentiation of *C. neoformans var. neoformans* and *C. neoformans* var. gattii. All those isolates identified as *C. neoformans* and gave negative test on Canavanine-Glycine-Brothymol Blue (CGB) Agar (Fig No:-6), indicating that the isolates were *Cryptococcus neoformans var. neoformans*.

Table4: Number of *C. neoformans* isolates against the number of samples examined.

Type of sample	Number of samples	Number of *C. neoformans* positive samples	Percentage of isolation
Cerebrospinal fluid	**40**	**14**	**35%**

The present retrospective study was undertaken to describe the disease in the HIV/AIDS infected population in terms of the immune status of the individuals, their standard of living, residential status either rural or urban, regularity of anti retroviral treatment, history of contact with birds or other animals, frequency and degree of contact with pigeon or their excreta, occupation and the nutritional status of the individuals. The data obtained either through the questioning to the patients or through the

hospital records, it was observed that among the 40 patients 21 was having the CD4 count less than 100 cells/ µl means they had the advanced stage of the disease indicating a highly significantly ($p<0.001$) lowered immune status (Table 5). Among the 14 isolates obtained from the 40 patients, 13 were obtained from these 21 patients who had this lower level of immunity which was again statistically significant (Table 6, Chart 1). These findings were almost similar to the findings of Walenkamp *et al.*, (2000) who also observed a positive relationship between the stages of HIV disease and the occurrence of Cryptococcosis. Persons who are in advanced stages of the disease are more prone to catch the infection easily.

Table 5: CD4 count of the patients during the time of sampling:

Patient no.	Stage of HIV	CD4 count (cells/µl)
1.	Stage 1	255
2.		350
3.		240
4.		390
5.		400
6.		460
7.	Stage2	240
8.		270
9.		285
10.		275
11.		300
12.	Stage3	155
13.		225
14.		140
15.		230
16.		180

Patient no.	Stage of HIV	CD4 count (cells/µl)
17.		150
18.		190
19.		250
20.	Stage4	95
21.		85
22.		70
23.		30
24.		46
25.		62
26.		75
27.		88
28.		94
29.		40
30.		78
31.		36
32.		93
33.		25
34.		37
35.		38
36.		24
37.		66
38.		74
39.		90
40.		82
41.		43

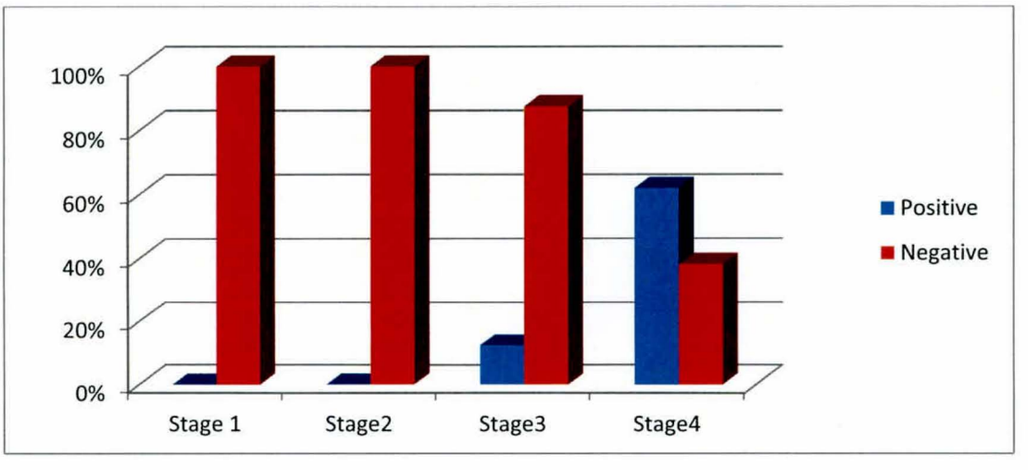
Highly significant p<0.001 by one way ANOVA

Table 6: Isolates obtained against different stages of the disease HIV/AIDS.

Stages of HIV/AIDS	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Stage 1	6	-	6
Stage 2	5	-	5
Stage 3	08	01	07
Stage 4	21	**13***	08

***Significant p<0.01**

Chart 1: Isolates obtained against different stages of the disease HIV/AIDS.



When the samples and the number of isolates were distributed according to the standards of living of the individuals (Table 7, Chart 2) and their residential status in terms of rural or urban (Table 8, Chart 3), no significant difference was observed ($p > 0.05$). No suitable record has been found to compare the finding of the present study. Although, persons leading an unhygienic lifestyle are proportionately more prone to infection.

Table 7: Number of isolates against the different Standards of living of the individuals

Standards of living	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Hygienic	07	01	06
Modrate Hygienic	08	02	06
Poorly Hygeinic	14	04	10
Unhygeinic	11	7	04

Non significant p>0.05

Chart 2: Number of isolates against the different Standards of living of the individuals

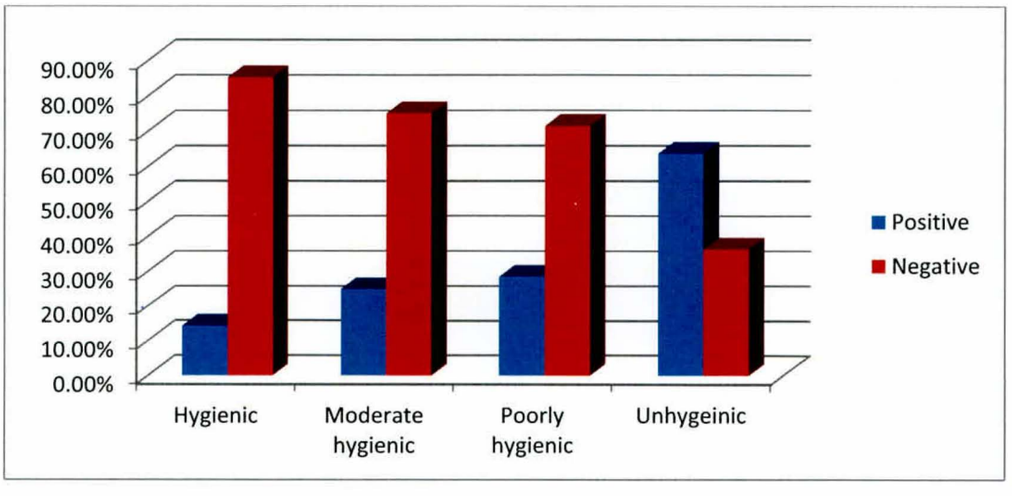


Table 8: Number of isolates against the residential area of the individuals

Residential area	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Rural	33	13	20
urban	07	01	6

Non significant p>0.05

Chart 3: Number of isolates against the residential area of the individuals

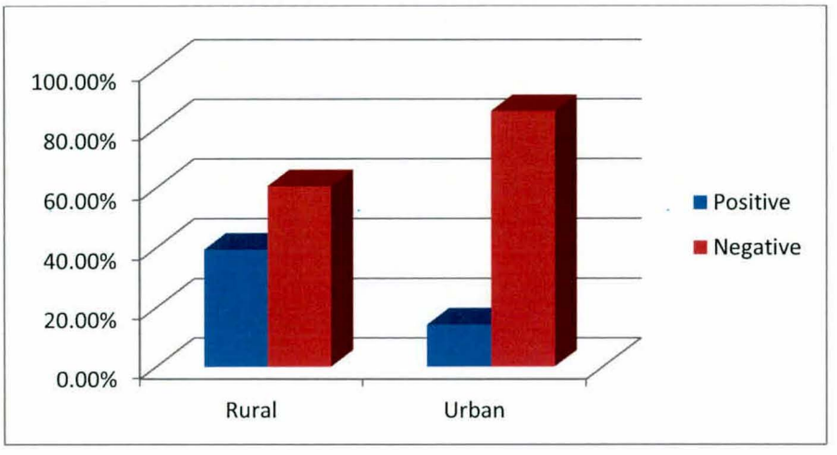
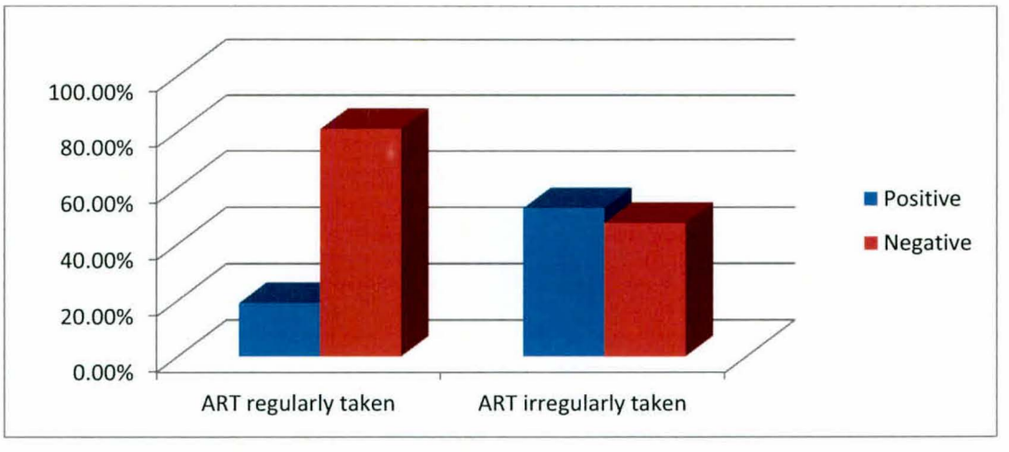


Table 9: Regularity of Anti Retroviral treatment against the isolates obtained.

Anti Retroviral treatment	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Regularly taken	21	04	17
Irregular	19	**10***	09

***significant p<0.05**

Chart 4: Regularity of Anti Retroviral treatment against the isolates obtained.



A significant difference ($p<0.05$) was observed in respect of the isolation of the organism between the two groups, one who took the

antiretroviral treatment regularly and the other who did not (Table 9, Chart 4). This may be due to the immunosupression of the individuals who did not follow the treatment regimen, which is again supported by the fall of CD4 count of those individuals. No suitable record has been found to compare the finding of the present study. But, according to Dromer *et al.,* (2004) and in the present study it has been observed that even a stage IV HIV positive patient is having a higher CD4 count when he or she is following the proper treatment schedule. This might be a reason that the earlier group is suffering less in Cryptococcosis than the later one.

Although there was no significant difference whether the individuals were in contact with the animals or birds in general (Table 10, Chart 5), but HIV/AIDS positive persons if come in close and frequent contact with pigeons there is a significantly ($p>0.05$) higher chances of development of Cryptococcosis (Table 11, Chart 6). This observation is strongly supported by the findings of Micalizzi *et al.,* (1997); Nosanchuk *et al.,* (2000); Shrestha *et al.,* (2004) and Lagrau *et al.,* (2005). In their studies they reported that bird excreta could be a potential source of infection for the human individuals. Therefore, the present study also suggests the immunocompromised persons to avoid both pet birds as well as the avian excreta.

Table 10: History of contact with birds and other Animals against the isolates obtained.

contact with birds and other Animals	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Only animal	05	-	05
Only bird	07	04	03
Both bird and animal	22	09	13
No contact	06	01	05

Non significant p>0.05

Chart 5: History of contact with birds and other Animals against the isolates obtained.

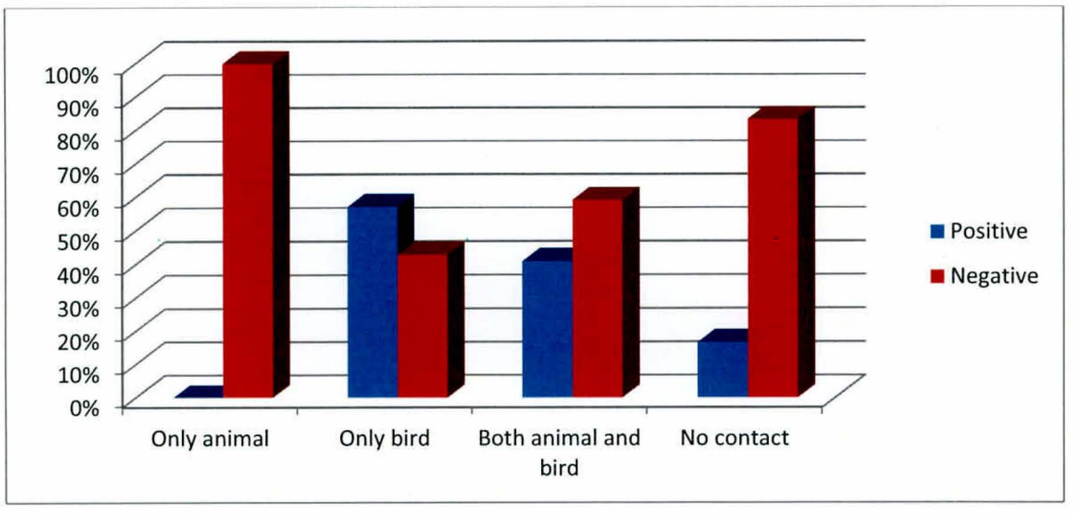


Table 11: Frequency and degree of contact with pigeons against the isolates obtained.

Frequency and degree of contact with pigeons	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Frequent and close contact	23	**12***	11
Occasional	11	01	10
Never	06	01	05

***significant p<0.05**

Chart 6: Frequency and degree of contact with pigeons against the isolates obtained.

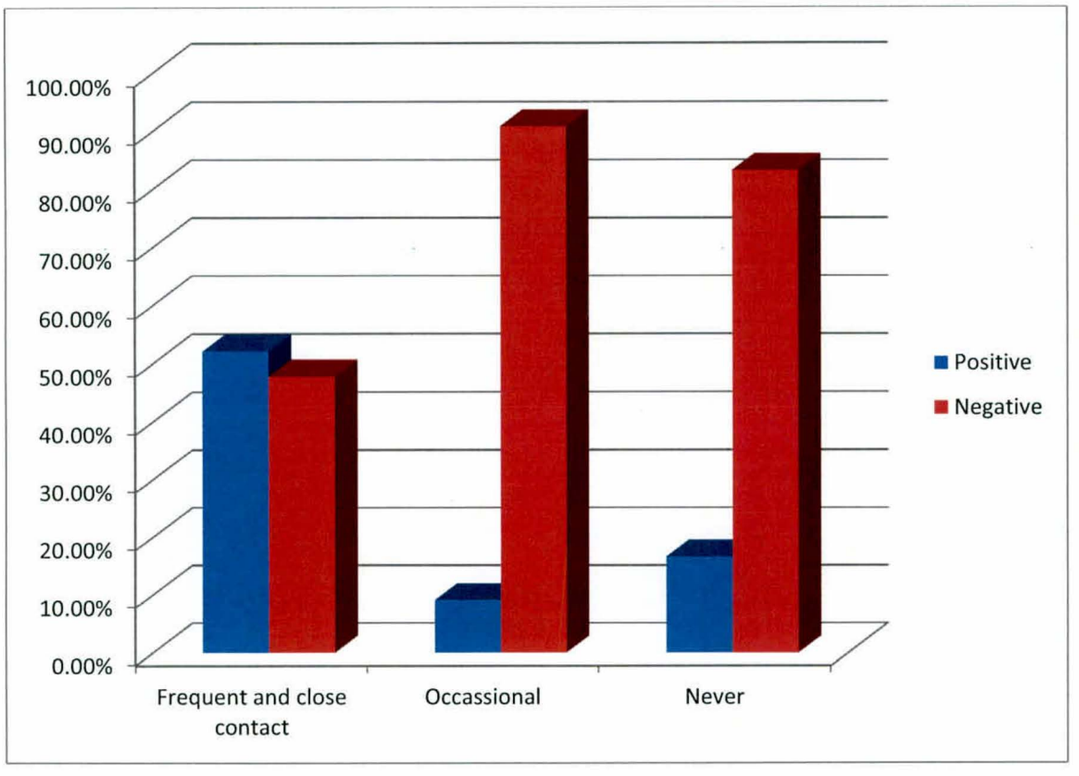


Table 12: Occupation of the HIV/AIDS patients against the isolates obtained

Occupation of the HIV/AIDS patients	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Only Animal Husbandary	05	-	05
Poultry Farming including Pigeon	23	12	11
Poultry Farming excluding Pigeon	06	01	05
Wood Cutter	04	01	03
Carpenter	02	-	02

Non significant p>0.05

Chart 7: Occupation of the HIV/AIDS patients against the isolates obtained

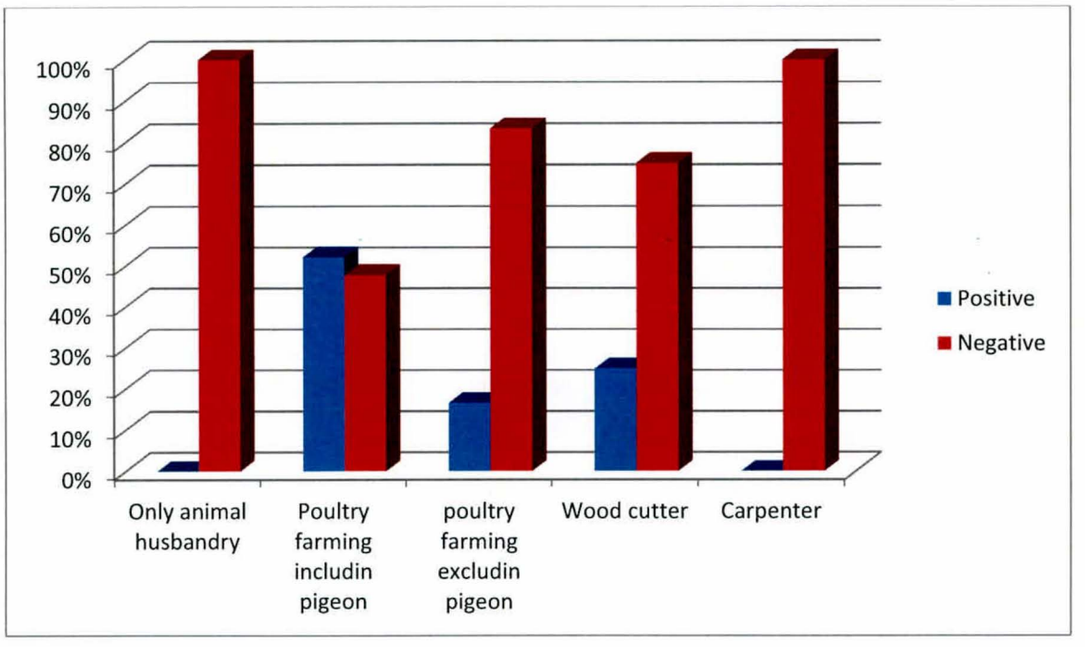
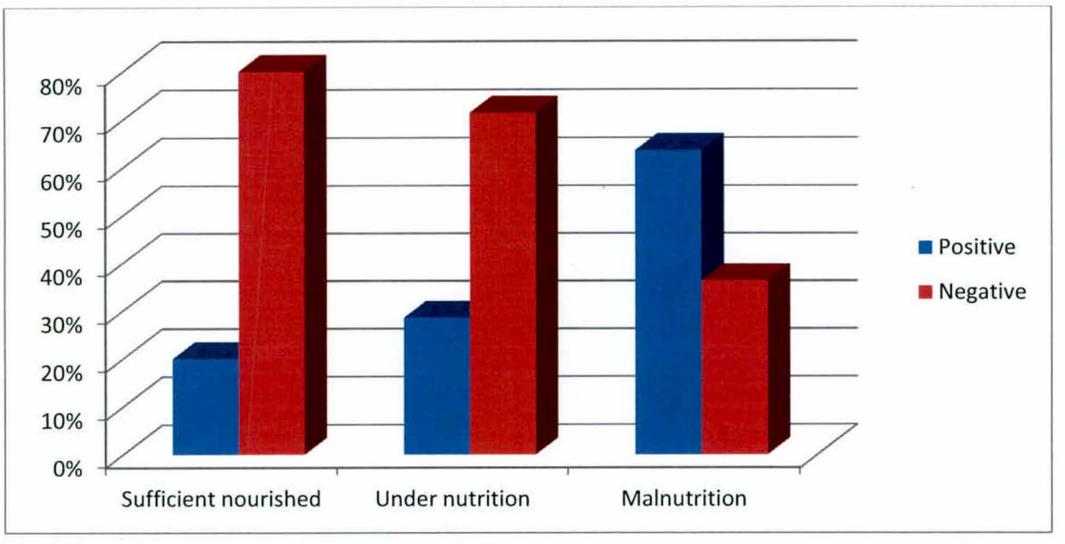


Table 13: Nutritional Status of the HIV/AIDS patients against the isolates obtained

Nutritional Status of the HIV/AIDS patients	No. of samples collected (n=40)	C. neoformans positive samples(n=14)	C. neoformans negative samples(n=26)
Sufficient Nourished	15	03	12
Under Nutrition	14	04	10
Malnutrition	11	07	04

Chart 8: Nutritional Status of the HIV/AIDS patients against the isolates obtained



No significant ($p>0.05$) difference was observed in the occurrence of the disease among the HIV/AIDS patients across their occupational difference, although a higher proportion (Table 12, Chart 7) of recovery of the pathogen was observed from the group who were engaged in poultry keeping including pigeon. The nutritional status of the individual was also of no significant difference in the predisposition of the disease in the HIV infected individuals (Table 13, Chart 8). No suitable record has been found to compare the findings of the present study in this regard.

ANTIFUNGAL SUSCEPTIBILITY PROFILE (AGAR DIFFUSION ASSAY):-

- Antifungal sensitivity pattern of all the 14 isolates of *C. neoformans* was tested against 5 common antifungal agents who are commonly used as therapy against this infection.
- Agar Diffusion Assay was adopted to determine the MIC values of Fluconazole, Ketoconazole, Itraconazole, Voriconazoleand Amphotericin-B by using MIC strips (Antifungal Ezy™ MIC Strips, Himedia®) (Fig: 11).

Table 14: In vitro response of five antifungal agents against fourteen (14) *Cryptococcus neoformans* isolates:-

No. of isolates	Antifungal Agents	MIC (µg/ml)	
		Range	MIC90
Cryptococcus neoformans (14)	Fluconazole	1-128	8
	Itraconazole	0.03-1	0.25
	Ketoconazole	0.03-2	0.125
	Voriconazole	0.03-2	0.125
	Amphotericin-B	0.03-4	0.5

- A total of 14 isolates were tested for their susceptibility against 5 important antifungal agents (Table: 14).
- Majority of the isolates were found susceptible to most of the drugs.
- Only exception was recorded in case of 2(14.3%) isolates, which were showing resistance to fluconazole (MIC value 64 µg/ml) and 1(7.1%) isolate was showing resistance to Ketoconazole (MIC value >0.5 µg/ml).

The observation of the present study was supported by the findings of Casadevall *et al.,* (1993); Paugam *et al.,* (1994); Datta *et al.,* (2003); Anuradha *et al.,* (2011) and Rajani *et al.,* (2014). In most of their observations, the authors found most of the clinical/environmental isolates susceptible to most of the commonly used antifungal agents. At the same time most of them also reported a kind of development of resistance for few isolates against few antifungal agents mostly Flukonazole. This kind of finding is of great concern as there are very limited numbers of effective antifungal agents for therapeutic use. So they should be used very cautiously and judiciously.

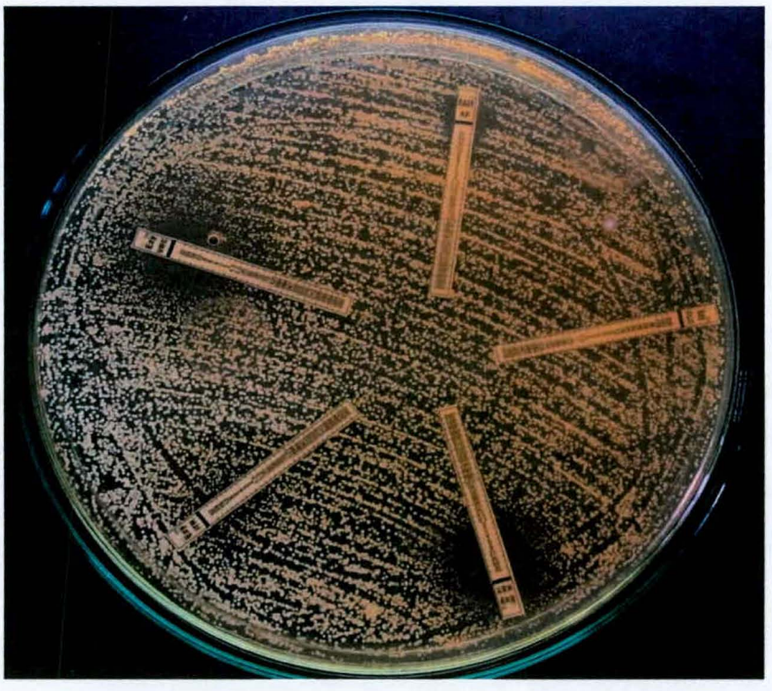


Figure:-11 Antifungal sensitivity testing of isolated *Cryptococcus*

RAPD analysis:-

The RAPD fingerprint profile of the 14 strains of *C. neoformans* obtained with the three different 10-mer primers are shown in figure15. Primer OPH-20 revealed major differences in banding patterns ranging between 1500 bp and 400 bp, which distinguished the profiles a, b and c (fig. 12). Primer OPH-02 revealed 3 different profiles in banding patterns ranging between about 2000bp and 400bp, which distinguished the profiles in three categories namely i, ii and iii (fig. 12). Primer R-28 revealed 3 different profiles in banding pattern ranging between about 1500bp and 200bp, which distinguished the profiles in 1, 2 and 3 categories (fig. 12). Table 15 summarises the RAPD profiles for all the isolates of *C. neoformans*, which generates 4 distinct RAPD finger print patterns (designated as pattern-I through pattern-IV) were identified by 3 primers. Of the 14 strains 4 were assigned to pattern I, 4 were assigned to pattern II, 3 were assigned to pattern III and the remaining 3 strains were assigned to pattern IV (Table 15). This result showed a wide genetic variability of *C.*

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSION

India is one of the major HIV/AIDS burdened countries. The incidence of Cryptococcosis, one of the important opportunistic pathogens in HIV/AIDS patients in India, particularly in Bihar State was not well-studied and therefore an epidemiological survey of these infection among these patients was being undertaken in the present study. Study of the zoonotic potentiality of the pathogen was also an important aspect. The present study was conducted in a period of 8 months ranging from January through August, 2015 with the samples collected from Jawaharlal Nehru Medical College and Hospital, Bhagalpur, Bihar. In a nutshell, the present study clearly demonstrates that Cryptococcus is a major opportunistic pathogen for the HIV positive individuals in general and the persons with progressive HIV in particular. The organism was recovered from 14(35.00%) out of 40 HIV/AIDS patients. Its occurrence is much more to the patients who do not follow the scheduled anti retro viral treatment. Persons who are in close and intimate contact with birds specially the pigeons and the avian excreta are more susceptible to the disease indicating a clear zoonotic potentiality of the pathogen. The standard of living, residential status in respect of rural or urban area, history of contact with other animals, nutritional status or occupation does not have much more significant effect on the occurrence of the disease in the HIV/AIDS patients in the study area. The present study also revealed the susceptibility pattern of the isolates against five important antifungal agents and pointed out the development of some sort of resistance by two isolates against Fluconazole and one isolate against Ketoconazole. The molecular study by using the RAPD with the isolates revealed the four different patterns of genetic diversity.

CHAPTER SIX

FUTURE SCOPE OF RESEARCH

The present study was carried out with a number of limitations. Therefore, the researcher felt that there are so many aspects of the disease which can be explored in future research work. The following aspects could not be covered in the present study:

- Large scale epidemiological study by using molecular tools to find out the genetic diversity of the pathogen in India.
- To study the ecological aspect of the disease in an extensive manner.
- Progressive epidemiological study to establish the zoonotic potentiality of the pathogen more reliably.
- Molecular identification of the pathogenic property of the pathogen.
- Genetic mapping of the pathogen isolated from different parts of the country.

CHAPTER SEVEN

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CHAPTER EIGHT

APPENDIX

APPENDIX

MEDIAS:-

PRIMARY MEDIA:-

1. **Bird Seed Agar(GuizotinaAbyssinica Creatinine Agar or Niger Seed Agar or Staibs' Agar)**Grams/lit(Himedia®):-It is used for selective isolation and differentiation of Cryptococcus neoformans from other Cryptococcus and other yeasts.

INGREDIENTS:

GuizotinaAbyssinica seed-70.00gms

Agar-20.0 gms.

Dextrose-15.0 gm.,

Creatinine-0.78 gm.

Chloramphenicol-0.05gm,

Final pH=-6.7± 0.2 at 25°C.

PREPARATION: Suspended 10.08 Grams in 99 ml distilled water.Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbspressure (121°C)for 15 minutes. Cool to 45°C and add 100 mcg diphenyl per ml of medium (1ml of sterile 1% w/v aqueous solution of diphenyl).

2. **Sabourad Dextrose Agar (Difco):**

Approximate formula per litre:

Peptic digest of animal tissue	5.0 g
Pancreatic digest of casein	5.0 g
Dextrose	40.0 g
Agar	15.0 g

65.0 g of the powder was suspended in 1 l of purified water. To mix it thoroughly, it was heated with frequent agitation and boiled for 1minute to completely dissolve the powder. Final pH adjusted to 5.6±0.2. Then the

media was autoclaved at 121^{0}c for 15 minutes at lbs. After autoclaving it was cooled and dispensed in tubes and / or in plates and allowed to be solid and stored in refrigerator.

3.Sabourad Dextrose Agar with Chloramphenicol (Himedia®):

Sabourad Dextrose Agar Base (Dextrose Agar Base, Emmons):

Standard formula:

Peptone special	10.00 gm. /lit
Dextrose	20.00gm/lit
Agar	17.00gm/lit
Final pH (at 25^{0}c)	7.0_+0.2

CC supplement:

Standard Formula: (per vial, sufficient for 500 ml medium)

Chloramphenicol	25.00 mg

23.5 g of the powder (A) was suspended in 500 ml of distilled water. It was heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Final pH adjusted to 7.0±0.2. Then the media was autoclaved at 121^{0}c for 15 minutes at 15lbs. After autoclaving it was allowed to cool to 45-50^{0}c. The contents of CC supplement vial (B) was rehydrated aseptically with 5 ml 0f 50% v/v acetone and then aseptically added to the prepared agar media (1 vial for 500 ml media) and mixed well. The media was dispensed in tubes and/or in plates and allowed to be solid and stored in refrigerator.

4.Corn Meal Tween 80 Agar (Himedia®):

Standard formula:

Corn meal, infusion from	125.00 gm/lit
Agar	50.00 gm/lit
Distilled water	3000 ml

Polysorbate(Tween)801%

17.0 g of the powder was suspended in 1 L of purified water. To mix it thoroughly, it was heated with frequent agitation and boiled for 1minute

to completely dissolve the powder and added 1% polysorbate(Tween 80). Final pH adjusted to 6.0±0.2. Then the media was autoclaved at 121^{0}c for 15 minutes at lbs. After autoclaving it was cooled and dispensed in tubes and / or in plates and allowed to be solid and stored in refrigerator.

5.Urea Agar Media (Himedia®):

Urea Agar Base (Christensen):

Standard formula:

Peptic digest of animal tissue		1.00 gm/lit
Dextrose	1.00 gm/lit	
Sodium Chloride		5.00 gm/lit
Disodium phosphate		1.20 gm/lit
Mono-potassium phosphate		0.80 gm/ lit
Phenol red		0.012 gm/lit
Agar		15.00gm/lit

Urea (40%) solution:

Formula (5ml per vial):

Urea	2.00 gm
Distilled water	5.00 ml

To make 1 lit media, 24.01 grams of urea agar base was suspended in 950 ml distilled water and mixed by boiling and then the final pH was adjusted to 6.8±0.2. Then the media was autoclaved at 121^{0}c for 15 minutes at 15 lbs. After autoclaving it is allowed to cool to 50^{0}c. The refrigerated urea solution was warmed up to room temperature and 50 ml of urea (5 vials) was added mixed well to the prepared media. The media was dispensed in tubes and/or in plates and allowed to be solid and stored in refrigerator.

6.1% PEPTONE BROTH(Himedia®):-

STANDARD PROTOCOL:

Ingredients:-

Peptic digest of animal tissue---10.00

Sodium Chloride.....5.00

pH at 25^{0}C=7.2 ± 0.2

Suspend 15.0 grams in 1000 ml distilled water.Then, mixed well and dispensed into tubes with or without inverted Durham's tubes and sterilize by autoclaving at 15 lbs. pressure at 121^{0}C for 15 minutes.

USE: It is used to observe clear, round, thick capsuled appears after inoculation.

7. Fermentation Test:-

Preparation:

Durhamtubes(12X150(or13X100))mmtesttubescontainssmall(6X50mm) Invertedtubes),caps.

Compoundstobetested

F1 Glucose	**F2 Glactose**	**F3 Maltose**	**F4 Fructose**
F5 Sucrose	**F6 Trehalose**	**F7 Manitol**	**F8 Lactose**
F9Negativeco			

Procedure:

(Fermentation of carbohydrates where fermentation means only production of gas and independent of pH change).For fermentation studies peptone water with phenol red (Himedia) was used (MacFaddin J.F., 1985) to study the ability of Cryptococcus species to ferment different carbohydrates (**Glucose, Glactose, Maltose, Trehalose, Fructose, Sucrose, lactose, Manitol**).

1% peptone water was prepared according to the protocol of the manufacturer (Himedia) and poured in test tubes (5ml of peptone water in each tube) containing inverted durham tubes. The tubes were then autoclaved at 121 ^{0}C and 15 lbs pressure for 15 minutes. After autoclaving a loopful of test culture was inoculated into each tube and then carbohydrate discs (25mg/disc)(Himedia) was added to the tubes (one disc in each tube) and incubated at 28^{0}C for 24-48 hours. After

incubation they were observed for the presence/absence of gas in the durham tubes indicating the ability/inability of the organism to ferment sugars. *C. neoformans* is unable to ferment sugars.

8. <u>Assimilation of Nitrogen Compounds:</u>

For Assimilation studies the medium was prepared by adding 0.675 grams Yeast Nitrogen Base (YNB)(Himedia) and 2mg Bromo Cresol Purpel (Himedia) to 100 ml of deionised water, heating to boiling, and autoclaving at 121 ^{0}C for 15 minutes. After cooling, 10 ml of media was poured in test tubes and different sugars discs (25mg/disc)(Himedia) was added (**Glucose, Glactose, Maltose, Trehalose, Fructose, Sucrose, lactose, Manitol**). Each tube of assimilation, media was inoculated with an inoculating needle from old isolate of *Cryptococcus neoformans* and incubated at 30^{0}C temperature and read at daily intervals for 96 hours. A positive reaction was indicated by growth on media and a change in colour of the indicator from purple to yellow.

<u>Ingredients (YNB)</u>:-grams/litre

Amonium sulphate---------5.00

L-Hitidine hydrochloride---0.01

DL-Methionine----------0.02

Dl-Tryptophan----------0.02

Biotin----------0.000002

Ca-pantothenate--------0.0004

Folic acid----------0.000002

Inositol----------0.002

Niacin----------0.0004

P-aminobenzoicacid(PABA)-0.0002

Pyridoxine Hcl----------0.0004

Riboflavin----------0.0002

Thiamine Hcl----------0.0004

Boric acid----------0.0005

Copper sulphate----------0.00004

Potassium iodide----------0.0001

Ferric Chloride----------0.0002

Megnasium sulphate-----0.0004

Na-molybdate----------1.00

Zinc sulphate----------0.01

Monoptasium phosphate-0.05

NaCl----------0.10

$CaCl_2$----------0.10

9. CANAVANINE-GLYCIN-BROMOTHYMOL BLUE AGAR(CGB):-

It is used for isolation of *Cryptococcus neoformans var. neoformans and var. gattii.*

TO MAKE 1 LITTER:-

MAKE UP SOLUTION A AND B IN ADVANCE.

SOLUTION A:

1. GLYCINE=10g
2. KH_2PO_4=1g
3. $MgSO_4$=1g
4. THIAMINE-HCL=1mg
5. L-CANAVANINE SULFATE=30mg
6. DISTILLED WATER=100 mL

Dissolve ingredients in small beaker and adjust P^H to 5.6.Filter sterilise solution using a 0.22μm filter membrane .Store at 4^0C until ready to use.

SOLUTION B:

1. Bromothymol Blue=0.4g
2. 64mL 0.01N NaOH.
3. Distilled Water=36 mL

Dissolve the Bromothymol Blue in NaOH and add to the water.

TO MAKE 1 LITER OF THE MEDIA MIX TOGETHER THE FOLLOWINGS:

1. Distilled Water =880 Ml

2. Solution B=20 mL
3. Agar=20 g

Autoclave to 121^{0}C (250^{0}F) for 15 minutes, cool to 50^{0}C.

Then, add 100mL of solution A And mix

Lastly, dispense into Petri-dishes. After, solidify inoculum is given and observe 48hrs-96hrs at 37^{0}C in the Incubator.

In case of *C. neoformans var. neoformans*, colour would not change (remain yellow) but *C. neoformans var. gattii* changed the colour of the media yellow to blue.

10. YEAST DNA ISOLATION KIT (Genei):

GeneiPure™ Yeast DNA Purification Kit **GeNei™**

Materials Supplied:

The list below provides information about the materials supplied in the kit. The components should be stored as suggested.

Materials	Quantity			Store
	612115800021730 (20 preps.)	612115800031730 (50 preps.)	612115800051730 (250 preps.)	
Yeast Lysis Buffer	12 ml	30 ml	150 ml	4°C
Proteinase K	0.5 ml	1.25 ml	1.25 ml x 5	-20°C
Lyticase	2000 U	5000 U	5000 U x 5	-20°C
RNase A	8 mg	20 mg	20 mg x 5	-20°C
Lysis Buffer I	4 ml	10 ml	50 ml	RT
Lysis Buffer II	4 ml	10 ml	50 ml	RT
Wash Buffer I (Concentrate)	2.5 ml	7 ml	35 ml	RT
Wash Buffer II (Concentrate)	2.5 ml	7 ml	35 ml	RT
Elution Buffer	4 ml	10 ml	50 ml	RT
GeneiPure™ Columns	20 Nos.	50 Nos.	250 Nos	RT
Collection Tubes	60 Nos	150 Nos	750 Nos	RT

Note: Room Temperature not to exceed 20-25°C.

Material required but not supplied:

Equipment : Microcentrifuge, Water Bath/Dry Bath, Vortex

Reagent : Absolute ethanol (96-100%), Glass Distilled Water (Sterile)

Other Requirements: 1.5 ml vials (Sterile)