SCREENING AND SELECTION OF *Cryptococcus Neoformans* USING UREASE TEST AS A VIRULENCE FACTOR

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CERTIFICATION

I certify that this work was carried out by Okungbowa Jennifer Imade of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State. Nigeria.

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EXTERNAL EXAMINER	**DATE**

DEDICATION

I dedicate this work to my heavenly father, the God Almighty who has kept me alive to see this blessed day, protecting and guiding me all along and my wonderful and ever-loving parents Mr. and Late Mrs. M. W. Okungbowa for all your unending love and care you have shown me and all the supports you have given me all through my academic year. Thank you very much.

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ABSTRACT

In this study, screening and selection of *Cryptococcus neoformans* was conducted using Urease test. Pigeon dropping is known to be a source of this infectious organism. A total of twelve (12) samples from pigeon droppings were collected from three markets in Benin City. All samples were cultured on SDB (Sabouraud Dextrose Broth) and incubated for 24hrs at 37°C. After 24hrs, the isolates were sub-cultured on fresh SDB and incubated for 24hrs. After growth was observed, the isolates were plated on SDA (Sabouraud Dextrose Agar) for identification of the organism. Isolates of the organism obtained from this samples that was sub-cultured were subjected to urease test. Clinical samples collected from UBTH were also subjected to urease test. The results showed that the isolates that were positive for urease tests with highest growth rate at 37°C were recorded for samples

obtained from Oliha market. This experiment shows the possibility of isolating and selecting *C. neoformans* on urease test.

CHAPTER ONE

INTRODUCTION

Microscopic fungi comprise a large number of microorganisms of very different characteristics. Among them, a few can cause disease in humans, especially among immunocompromised patients (Brown GD, Denning DW, Gow NA, et al. 2012). Opportunistic invasive fungal diseases have become a problem of concern for the national health systems worldwide due to their high associated mortality and economical cost (Drgona L, Khachatryan A, Stephens J, et al. 2014), (Menzin J, Meyers JL, Friedman M, et al. 2011). The main human fungal pathogens belong to the genera Candida, Aspergillus, and Cryptococcus. All of them are causes of disease in immunosuppressed patients, so they are considered opportunistic pathogens.

The mechanisms involved in invasive fungal disease are different and involved both fungal and host elements. Some of pathogenic mechanisms are shared by all the fungal pathogens, such as the ability to grow at physiological temperature, resistance to the immune challenges (such as free radicals), and others. But the case of Cryptococcus offers an excellent model to investigate fungal pathogenesis for several reasons.

Few of these fungi are pathogenic to humans. Of these, *Cryptococcus neoformans* has emerged as an important cause of mortality in immunocompromised patients,

especially those with AIDS. As a result, extensive research efforts have addressed the pathogenesis and virulence of this organism. It is a spherical, encapsulated, non-myceliated, non-fermenting yeast like fungal cell (Okagaki et al., 2010). It is pathogenic to humans and animals. It also causes fatal meningitis primarily in immunosuppressed individuals (Cogliati, 2012). The organism exists as both free living and in association with other variety of hosts (Olszewski et al., 2010). *C. neoformans* is a basidiomycetous fungus that is ubiquitous in the environment, where it is found in soil, in association with certain trees, and in bird guano (Casadevall, A., and J. R. Perfect. 1998). Because of its ubiquity, it has been suggested that most people are exposed to *C. neoformans* early in life (Goldman, D. L., H. Khine, J. Abadi, D. J. Lindenberg, L. Pirofski, R. Niang, and A. Casadevall. 2001.).

Cryptococcus neoformans has been known as causative agent of disease since the 19th century (Heitman J, Kozel TR, Kwon-Chung KJ, et al. 2011). However, its incidence raised significantly at the end of the 20th century, associated with the emergence of HIV infection, and it was estimated that it could affect around 10% of AIDS patients. The introduction of the antiretroviral therapy has controlled the incidence of cryptococcosis in developed countries, but it is still a major concern in developing areas. The fungus is heterothallic, with mating types MATa and MATα. It is also called an opportunistic pathogen that primarily infects immune-

compromised individuals. Its metabolism is mainly respiratory, so its growth is highly dependent on the presence of oxygen (Casadevall A, Perfect JR. 1998).

Infection is generally initiated by inhalation of basidiospores or poorly encapsulated yeast cells and is usually contained by granulomatous inflammation within the lung in immunocompetent individuals which is first colonized by the pathogen. The immune response of this organ is very specific because it is continuously exposed to exogenous particles (Martin TR, Frevert CW. 2005), and in fact, it is very effective at controlling the dissemination of the cryptococcal cells.

However, in immunosuppressed patients, in particular those defective in CD4 T cells, *C. neoformans* can replicate and disseminate through the organism. The most characteristic clinical outcome appears when it reaches the brain, where it causes meningoencephalitis. This disease is very serious and has a high mortality associated (around 20–50% of affected patients).

In immune compromised hosts, the fungus is frequently capable of disseminating into the bloodstream and then crossing the blood-brain barrier to cause meningoencephalitis. (Lee, S. C., A. Casadevall, and D. W. Dickson. 1996). *C. neoformans* has emerged as a significant opportunistic pathogen because of the pandemic of AIDS and the widespread use of immunosuppressive therapy; this fungus now represents the most common cause of fungal infections of the central

nervous system (CNS) (Perfect, J. R., and A. Casadevall. 2002.). CNS cryptococcosis is fatal without treatment, and therapy with the antifungal drug amphotericin B has limitations due to potential host toxicity. Treatment failures with death in the first 3 months after diagnosis still range between 10 and 25%. The importance of identifying new targets for antifungal therapy is emphasized by a recent outbreak of *C. neoformans* infections on Vancouver Island in British Columbia, Canada. None of the approximately 60 cases involved people coinfected with human immunodeficiency virus, and the disease occurred primarily in immunocompetent individuals (Stephen, C., S. Lester, W. Black, M. Fyfe, and W. Raverty. 2002).

Asexual reproduction takes place either by budding or, in the case of MAT cells, by haploid fruiting in response to nutrient deprivation or exposure to the mating pheromone a factor (Wickes, B. L., M. E. Mayorga, U. Edman, and J. C. Edman. 1996). Sexual reproduction occurs when cells of opposite mating types come together to form a heterokaryon that ultimately leads to the production of basidia and basidiospores (Kwon-Chung, K. J. 1975). Desiccated cells and the spores formed by haploid fruiting or sexual reproduction have all been suggested to serve as infective particles, which must be less than 2 m in diameter to penetrate the lung parenchyma (Hatch, T. F. 1961), (Rodrigues, M. L., C. S. Alviano, and L. R. Travassos. 1999). In most immunocompetent individuals, this infection is either

cleared or remains dormant until an immune imbalance leads to further development. In the setting of compromised immune function, however, the fungus disseminates, with particular tropism for the central nervous system. In severe cases, cryptococcal infection progresses to a meningoencephalitis that is fatal if left untreated. The characteristic neurotropism of *C. neoformans* was first recognized in 1914 by (Verse´ 1914), and 2 years later by (Stoddard and Cutler 1916).

However, Stoddard and Cutler called the etiologic agent Torula histolytica and the disease "torulosis" by misinterpreting the fungal capsule as evidence of fungal histolytic action in the host tissue (Kwon-Chung and Bennett 1992). Confusion about the identity of the cryptococcosis agent persisted until Benham performed comprehensive studies with clinical *Cryptococcus* strains and concluded that all of the strains from human infections belonged to one species with two varieties based on serological differences (Benham 1935, 1950). She proposed to replace "torulosis/torula meningitis" with cryptococcosis and to conserve the fungal name *C. neoformans* (Benham 1950). Cryptococcal antigenic heterogeneity was confirmed in 1950 by Evans who identified three serotypes: A, B, and C (Evans 1950). A fourth serotype, D, was discovered in 1968 (Wilson et al. 1968).

The Fungus exists as these four serotypes (A, B, C and D). Serotypes A and D are responsible for the vast majority of cases of cryptococcosis worldwide, and thus account for more than 90% of clinical isolates. Serotypes B and C affect patients

with no apparent immunological defects, and are more present in tropical and sub-tropical regions – although a recent outbreak of cryptococcosis on Vancouver Island (Canada) indicates that these serotypes can adapt to different environmental conditions (Stephen, C., Lester, S., Black, W., Fyfe, M. and Raverty, S. 2002). Most susceptible to infection are patients with T-cell deficiencies (Kwon-Chung KJ. 1992), (Mitchell TG, Perfect JR. 1995). *C. neoformans* causes most cryptococcal infections in humans, so this review will focus on information from the neoformans variety of this basidiomycetous fungus.

C. neoformans is found worldwide; its main habitats are debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings (Kwon-Chung KJ. 1992), (Rippon JW. 1988). Not part of the normal microbial flora of humans, *C. neoformans* is only transiently isolated from persons with no pathologic features (Mitchell TG, Perfect JR. 1995), (Duperval B, Hermans PE, Brewer NS, Roberts GD. 1977). It is generally accepted that the organism enters the host by the respiratory route in the form of a dehydrated haploid yeast or as basidiospores. After some time in the lungs, the organism hematogenously spreads to extrapulmonary tissues; since it has a predilection for the brain. (Kwon-Chung KJ. 1992). *C. neoformans* virulence is mediated predominantly by a polysaccharide capsule that surrounds its cell wall and has multiple effects on the host immune system. This structure provides a physical barrier that interferes with normal

phagocytosis and clearance by the immune system. Capsule components inhibit the production of proinflammatory cytokines, deplete complement components (by efficiently binding them), and reduce leukocyte migration to sites of inflammation (Buchanan, K. L., and J. W. Murphy. 1998.).

C. neoformans occurs ubiquitously in the environment and causes opportunistic infections in patients who are deficient in cell-mediated immunity. Most *C. neoformans* disease is caused by serotype A strains (Xu J, Vilgalys R, Mitchell TG. 2000). Although *C. neoformans* came to broad attention as the cause of an AIDS-defining illness (extrapulmonary cryptococcosis), in recent years disease in HIV-negative patients has increased and presents new clinical challenges (Chayakulkeeree M, Perfect JR. 2006). Although the disease is a worldwide threat, underdeveloped countries experience particularly high mortality rates. Current antifungal-based therapies against cryptococcosis are often incapable of completely eliminating the pathogen, leading to the recurrence of the disease (Bicanic and Harrison, 2004). Thus, the development of novel therapeutic strategies is paramount, but depends on an improved understanding of *C. neoformans* biology and pathogenesis. Together with Candida albicans and Aspergillus spp., *Cryptococcus neoformans* is one of the three leading causes of morbidity and mortality associated with fungal infections worldwide.

The generation of immunologic tools to fight cryptococcosis has been pursued for a long time through a variety of approaches (Devi, S. J. 1996), (Larsen, R. A., P. G. Pappas, J. Perfect, J. A. Aberg, A. Casadevall, G. A. Cloud, R. James, S. Filler, and W. E. Dismukes. 2005). Considering the premises on which to build active and/or passive vaccination, Levitz and collaborators have pointed out the pivotal role of a cell-mediated immune response in fighting cryptococcosis (Dan, J. M., and S. M. Levitz. 2006), (Levitz, S. M., and C. A. Specht. 2006), while Casadevall and Pirofski have emphasized the importance of humoral responses in protection against cryptococcal disease (Casadevall, A., and L. Pirofski. 2005), (Casadevall, A., and L. A. Pirofski. 2006).

1.1 Cryptococcus as environmental pathogen

When compared to other fungal pathogens, *C. neoformans* is very characteristic in the infection route and in the disease caused. For examples, other yeasts, such as Candida spp, are rarely acquired by inhalation and *C. neoformans* resembles more the infection caused by filamentous fungi or primary fungal pathogens, such as Histoplasma capsulatum. *Cryptococcus neoformans* has developed some virulence mechanisms that allow the survival in the lung and dissemination to the brain. Interestingly, some of them are also used to infect and cause disease in environmental host (Casadevall A, Steenbergen JN, Nosanchuk JD. 2003).

This yeast has a worldwide distribution and it can be disseminated using some birds as carriers (Littman ML, Borok R. 1968). It is believed that the continuous exposure to environmental stress, such as temperature fluctuations and dehydration has selected cryptococcal strains with a higher fitness in mammalian individuals.

Furthermore, it has the ability to infect a large number or organisms, such as amoebas, flies, nematodes, Lepidoptera, and even plants as Arabidopsis thaliana (Neilson JB, Ivey MH, Bulmer GS. 1978), (Steenbergen JN, Shuman HA, Casadevall A. 2001), (Apidianakis Y, Rahme LG, Heitman J, et al. 2004), (Mylonakis E, Moreno R, El Khoury JB, et al. 2005), (Warpeha KM, Park YD, Williamson PR. 2013). Even in the case of mammals, there are reports of infections in a wide range of animals, such as koalas, dolphins, and cats (Venn-Watson S, Daniels R, Smith C. 2012), (Malik R, Martin P, Wigney DI, et al. 1997). These multiples interactions are believed to be important for the virulence of *C. neoformans*, because the mechanisms that allow the fungal survival after interaction with these different hosts have selected multiple traits that can be used to adapt and cause disease in humans. Maybe the phenomenon that is best characterized is its ability to survive after the interaction with environmental predators, such as amoeba (Steenbergen JN, Casadevall A. 2003), (Casadevall A. 2012). This cryptococcal ability is very similar to the behavior during the

interaction with mammalian phagocytic cells. To positively be classified as a pathogen, an organism must be able to cause infection under certain conditions.

By this definition, *C. neoformans* can certainly be classified as a pathogen. Because the immunodeficient are more susceptible than the immunocompetent to infection with this yeast-like organism, *C. neoformans* is frequently referred to as an opportunistic pathogen. The factors that make *C. neoformans* a pathogen can be divided into two major groups. The first comprises the basic characteristics needed to establish an infection and survive in the human host; the second comprises the virulence factors that affect the degree of pathogenicity.

1.2 Urease

C. neoformans produces several degrading enzymes, such as proteases and lipases as virulence factors. These enzymes are also produced by other microbial pathogens, including fungi and bacteria (Schaller M, Borelli C, Korting HC, et al. 2005), (Singh G, Singh G, Jadeja D, et al. 2010), (Toth R, Toth A, Vagvolgyi C, et al. 2017). In the case of *C. neoformans*, there is another degrading enzyme, urease, which also plays a role during infection.

Urease catalyzes the degradation urea into CO_2 and ammonia and is required for nitrogen utilization in multiple organisms. *Cryptococcus neoformans* produces very high amounts of urease, and its presence has been used as a diagnostic tool for

cryptococcosis (Zimmer BL, Roberts GD. 1979). Urease is considered a virulence factor. Absence of this enzyme results in a fitness defect at slightly basic pH (Fu MS, Coelho C, De Leon-Rodriguez CM, et al. 2018). During infection, urease is required for brain invasion (Cox GM, Mukherjee J, Cole GT, et al. 2000). Urease promotes sequestration of cryptococcal cells at microcapillary vessels (Olszewski MA, Noverr MC, Chen GH, et al. 2004), and it has been hypothesized that ammonia promotes adhesion of *C. neoformans* either by increasing the expression of adhesins on the endothelia or by a direct toxic effect on the integrity tight junctions of the brain blood barrier (BBB) that would facilitate the brain invasion (Olszewski MA, Noverr MC, Chen GH, et al. 2004), (Taylor-Robinson SD, Jackson N, Buckley C. 1997). In agreement, proteins required for urease activity are also defective in brain invasion (Singh A, Panting RJ, Varma A, et al. 2013).

Although there have been some rare case reports of urease-negative *C. neoformans* strains causing human infection (Bava, A. J., R. Negroni, and M. Bianchi. 1993), (Ruane, P. J., L. J. Walker, and W. L. George. 1988), the vast majority of clinical isolates produce large amounts of urease. In fact, the rapid detection of urease activity is one means of tentatively identifying *C. neoformans* from clinical specimens (Canteros, C. E., L. Rodero, M. C. Rivas, and G. Davel. 1996), (Zimmer, B. L., and G. D. Roberts. 1979). It is likely that the primary role of this enzyme for *C. neoformans* is to convert urea to a usable nitrogen source in its ecological niche.

However, by analogy to the findings with some bacteria, this enzyme may be important for the ability of this yeast to survive within mammalian hosts.

Urease test is a routine test in the identification of *C. neoformans* and it has also been linked with virulence in this fungus (Fu et al., 2018). In the bacteria Helicobacter pylori and Proteus mirabilis, urease test has been found to be an important factor in pathogenicity of these organisms (Eaton et al., 1991; Tsuda et al., 1994). With this background, i decided to use urease test in selection of pathogenic *C. neoformans*. Probably, this particular factor may be able to differentiate between pathogenic and non-pathogenic *C. neoformans*.

1.3 Justification

Urease is one of the most important virulence factor of *C. neoformans*. This pathogenic organism produces very high amounts of urease, and its presence has been used as a diagnostic tool for cryptococcosis.

The aim of this work was:

i. To study the pathogenicity of the organism *Cryptococcus neoformans*.
ii. To isolate and carryout selection of *Cryptococcus neoformans* using urease test.

iii. To starve the organism of nitrogen nutrients using YEPD (Yeast Extract Peptone Dextrose).

CHAPTER TWO

MATERIALS AND METHODS

The materials used in this study includes; Petri dishes, McCartney bottles, Conical flasks, Measuring cylinder, Beaker, Eppendorf, Pasteur pipette, Bunsen burner, Incubator, Refrigerator, Pressure pot, Measuring scale, Shaker glass rod, Funnel, Inoculating loop, Cotton wool and Gas cylinder with a burner affix on top.

2.1 Description of Materials Used

Petri Dishes

A petri dish is a shallow transparent lidded dish, usually cylindrical with diameter mostly ranging from 30 to 200mm. In this work the 90mm plastic petri dishes were used.

McCartney Bottles

McCartney bottles are a set of thick, clear wide mouthed glass bottles made of borosilicate glass and aluminum caps.

Conical Flask

A conical flask is a type of laboratory flask with a flat bottom, a conical body and a cylindrical neck.

Beaker

A beaker is a cylindrical container usually made of glass with a flat bottom. Most beakers have a small spout (or beak). They are usually calibrated and range in sizes.

Pasteur Pipette

Pasteur pipette is a glass or plastic tubes that are tapered to a narrow opening point at the lower end and fitted with a plastic or rubber bulb at the upper end.

Measuring Cylinder

A measuring cylinder is laboratory equipment with a narrow cylindrical shape. It has a base for standing erect and is graduated along its length.

Shaker glass rod

The shaker glass rod is cylindrical glass equipment usually between 10 to 40 cm in length and about half a centimeter in diameter.

Pressure Pot

Pressure pots are pots made from metal pan, with a lid that has a locking device button. It has handles made of plastic on both sides for carrying the pot.

Measuring Scale

The measuring scale used is AWS Gemini 20 portable milligram scale. The scale is calibrated to three decimal places and can weigh from 1mg to 20mg.

Gas cooker

The gas cooker is a gas cylinder with a burner affix on top of it. The gas cylinder is about rated 9kg (which means it can hold up to 9kg of gas. The gas used was methane or cooking gas. The burner was made of metal and had a control valve.

Incubator

An incubator is an equipment that maintains optimal temperature, humidity and other conditions such as the CO_2 and O_2 content of the atmosphere inside.

Refrigerator

A refrigerator is an equipment that transfers heat from its inside to its external environment so that its inside is cooled to a temperature below the room temperature.

2.2 Use of Material

Petri dishes was used to hold solid growth medium for the culture of *Cryptococcus neoformans*. McCartney bottles was used to hold liquid and solid mediums for the culture of the microorganisms. Conical flasks were used to prepare and sterilize culture media and also to hold and store other liquid materials. Beaker was used to prepare culture media and to mix culture media during preparation before dispensing into conical flasks for sterilization. It was also used and can be used to store substances in the laboratory. Measuring cylinder was used to measure the liquid materials (solvent) in the laboratory. Measuring Scale was used to measure

the quantity of solid materials required for use in the laboratory. Pasteur pipette was used for inoculation and to measure small quantity of liquid (3ml and below) usually liquid culture media but may be used to dispense other liquid also. Workbench was where all the experiment was conducted. Cotton wool was used to swab/clean the work area and to wash bottles for reuse. Inoculating loop was used to inoculate samples to liquid media and also to streak solid media. Incubator was used to provide optimal growth environment by providing constant temperature and light condition necessary for growth. Refrigerator was used to cool media, store media and reduce the rate of microbial growth by lowering the temperature of the media/isolates. Pressure pot was used to sterilize glass wares and mediums by autoclaving. Funnel was used during dispense of liquid to avoid spill. Shaker glass rod was used to mix medium and also to stir mixture.

2.3 Sterilization of Materials

All glass wares were sterilized by autoclaving at 15 psi (121^0C) for 15 minutes. Plastic wares were sterilized by cleaning them with bleach or absolute ethanol. Inoculating loop was sterilized by flaming it till it is red hot. Petri dishes was purchased already sterile.

Sterilization of Workbench

The workbench was sterilized using bleach solution and burning of absolute (99.9%) ethanol. Cotton wool was soaked in bleach solution and used to clean the

workbench surface thoroughly. The edges and corners were also cleaned. An ethanol lamp consisting of a glass container, an aluminum cap and stove wick was used to sterilize the air by burning ethanol for 15 minutes.

2.4. Growth Media

The culture media used for this work includes: Sabouraud Dextrose Broth, Sabouraud Dextrose Agar and Urea Base Agar.

Sabouraud Dextrose Broth

Sabouraud dextrose broth has in composition per liter Peptone 10g; the carbon source is glucose 40g and distilled water.

Measure the composition into a beaker, add some distilled water and dissolve properly. Pour into a measuring cylinder and make it up to 1000ml. Dispense into conical flasks and sterilize by autoclaving at 121 ^{0}C for 15 minutes.

Sabouraud Dextrose Broth was used to culture the organism (*C. neoformans*) from the sample and also to subculture from plate.

Sabouraud Dextrose Agar

Sabouraud Dextrose Agar has in composition per liter Peptone (10g); Agar powder (15g); the source of carbon is glucose (40g) and distilled water (1000ml).

Measure the composition into a beaker, add distilled water and dissolve properly. Pour into a measuring cylinder and make it up to 1000ml. Dispense into conical flasks and sterilize by autoclaving at 15 psi (121 ^{0}C) for 15 minutes.

Sabouraud Dextrose Agar was used to identify the organism morphologically.

Urea Base Agar

Urea Base Agar has in composition per liter; Sodium chloride (5.00g), Disodium phosphate (1.20g), Monopotassium phosphate (0.80g), Peptone (1.00g), carbon source is glucose (1.00g), Agar powder (15.00g), Phenol red (0.012g), 40% sterile urea solution (50ml), pH at 6.8 ± 0.2 at 25 ^{0}C.

Measure the composition and dissolve in distilled water, make it up to 950ml/liter. Gently heat the medium to boiling with gentle swirling until the medium dissolves completely. Sterilize by autoclaving at 15psi (121 ^{0}C) for 15 minutes. Cool to 50^{0}C and aseptically add 50 ml of 40% sterile urea solution mix well and dispense into sterile tubes. Set in a slanting position. Do not reheat the medium as urea decomposes very easily.

Urea Base Agar was used to screen the organism based on the change in coloration observed.

2.5 Collection of Samples

A total of 12 environmental and 5 clinical samples were collected and screened. The environmental samples were obtained from three different markets in Benin

City (Uselu market, New Benin market and Oliha market). The samples were collected using sterilized McCartney bottles and a spatula. The spatula was used to pick up the samples (pigeon droppings) from cages of pigeons in each market and then dropped into the McCartney bottles. The bottles were sealed immediately and the spatula cleaned with cotton wool doused in methylated spirit. The clinical samples were obtained from the University of Benin Teaching Hospital (UBTH), Benin City. The samples were blood samples collected from persons screened and confirmed to be HIV/AIDS patients by lab attendants and put in sample bottles.

2.6. Innoculation

All samples were cultured on Sabouraud dextrose broth (SDB) for 24 hours at 37^0C. Inoculation was done using an inoculating loop for the environmental samples. A portion of the sample was picked with the inoculating loop and placed on Sabouraud Dextrose Broth (SDB) and then incubated for 24 hours at 37 ^{0}C. Inoculation was also carried out using a Pasteur pipette for the clinical samples. A specific quantity of the sample was picked and introduced into SDB and then incubated for 24 hours at 37 ^{0}C.

After 24 hours the inoculum was sub-cultured on fresh SDB and incubated for 24 hours after which growth was observed. After serial dilution was carried out, the

resulting isolates were then plated on Sabouraud Dextrose Agar (SDA) for identification.

2.7 Identification and Screening

The samples were identified on SDA using their morphological characteristics. Samples that showed white to milk mucoid colonies were picked/selected and inoculated on SDB (Sabouraud Dextrose Broth). Identification at this point was done with the physical eyes.

2.8 Urease Test

The samples were screened and selected using UBA (Urea Base Agar). The medium was prepared and poured in McCartney bottles to make slants. The slants were made by suspending the bottles containing UBA in a slanting position such that at the bottom there is sufficiency of the medium and along the length there is a smooth slant tapering towards the tip of the bottle. It was left for about 2 hours to gel after which the inoculum was dropped using a dropper pipette at the tip of the slant and the slants, incubated at room temperature (27 ^{0}C) for 72 hours. Observation was made for color change. The initial color of the medium was yellow before incubation at 27°C. A color change from yellow to pink after

incubation for a day indicates urease enzyme activity where urea is hydrolyzed to ammonia and carbamate (Casadevall and Steenberg, 2003).

Plate 1: Sabouraud dextrose broth in a mccartney bottle

Plate 2: Sabouraud Dextrose Agar on plate (Petri dish)

Plate 3: Slant of Urea Base Agar in a McCartney bottle

CHAPTER THREE

RESULTS

In this study, twelve (12) environmental samples were collected from three different markets and tested for the presence of *C. neoformans*. Five (5) Clinical samples were also collected from UBTH (University of Benin Teaching Hospital) and tested for the presence of *C. neoformans* in Urease test.

The isolates were cultured on SDA (Sabouraud Dextrose Agar) and production of a milk coloration was observed after incubation for 24hrs at 37°C. The resulting isolates were tested on slant UBA (Urea Base Agar) and then incubated for 24hrs to 72hrs at room temperature of 37°C. Growth observation was graded as 1+ to 3+ indicating the increased pink colour intensity after incubation from 24hrs to 72hrs.

Table 1 shows the number of isolates for *C. neoformans* that was present and absent on plated SDA (Sabouraud Dextrose Agar). Plate 4 shows isolates of *C. neoformans* on SDB (Sabouraud Dextrose Broth). Plate 5 shows the presence of *C. neoformans* isolates on plated SDA (Sabouraud Dextrose Agar). Table 2 shows the results of environmental isolates of *C. neoformans* that were positive and negative using urease test assay. Some of the samples from each of the markets was negative but the highest number of isolates was obtained from samples collected from Oliha market.

This signifies that *C. neoformans* is mostly present on sample collected from Oliha market. Table 3 shows the results of clinical isolates of *C. neoformans* that were positive and negative using urease test. The results gotten from environmental isolates and clinical isolates signifies that *C. neoformans* is mostly present on samples collected from the Hospital than samples collected from the market. Plate 6 shows the presence of *C. neoformans* isolates on UBA (Urea Base Agar).

Table 3.1: Isolates of *C. neoformans* that were present and absent on plated SDA (Sabouraud Dextrose Agar).

Site name	Sample/Isolates	Presence of *C. neoformans*
Uselu Market	Use1	+
Uselu Market	Use2	-
Uselu Market	Use3	+
Uselu Market	Use4	-
New Benin Market	NBM1	-
New Benin Market	NBM2	-
New Benin Market	NBM3	+
New Benin Market	NBM4	+
Oliha Market	Oliha1	+
Oliha Market	Oliha2	+
Oliha Market	Oliha3	+
Oliha Market	Oliha4	-

Note: + represents isolates that were present
- represents isolates that were absent

Plate 4: McCartney bottles containing isolates of *C. neoformans* on SDB (Sabouraud Dextrose Broth).

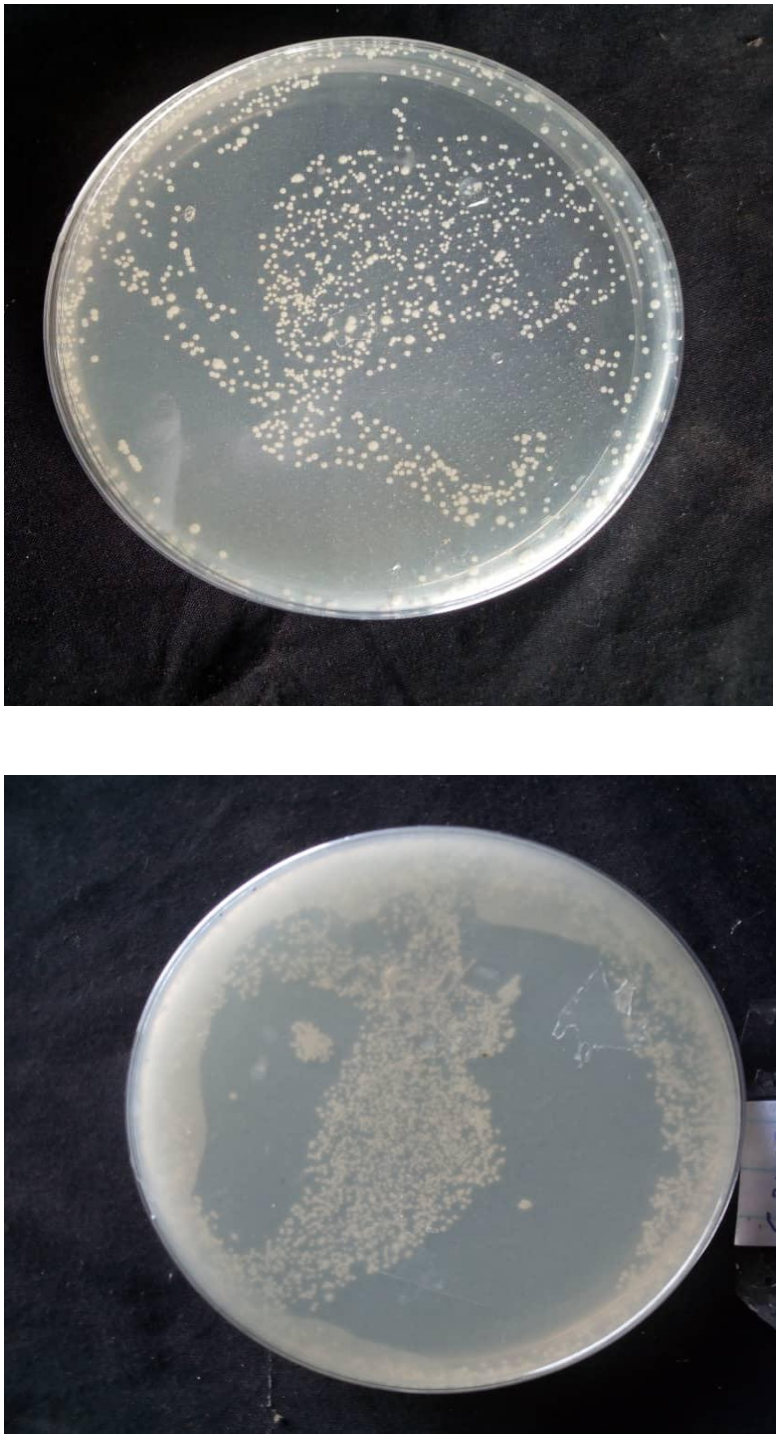

Plate 5: Presence of *C. neoformans* isolates on plated SDA (Sabouraud Dextrose Agar).

Table 3.2: Environmental isolates of *C. neoformans* that were positive and negative using urease test assay.

Site name Isolation	Sample/Isolates	1st Isolation	2nd Isolation	3rd
		24Hrs	48Hrs	72Hrs
Uselu Market	Use1	++	+++	+++
Uselu Market	Use2	-	-	+
Uselu Market	Use3	+	++	+++
Uselu Market	Use4	-	-	-
New Benin Market	NBM1	-	-	+
New Benin Market	NBM2	-	-	-
New Benin Market	NBM3	+	+	++
New Benin Market	NBM4	++	+++	+++
Oliha Market	Oliha1	+	++	+++
Oliha Market	Oliha2	++	+++	+++
Oliha Market	Oliha3	+	+++	+++
Oliha Market	Oliha4	-	-	+

Note: + represents isolates that were positive
- represents isolates that were negative
- No visible change
+ Slightly dense
++ Dense
+++ Very dense

Table 3: Clinical isolates of *C. neoformans* that were positive and negative using urease test assay.

Site name	Sample/Isolates	1st Isolation 24Hrs	2nd Isolation 48Hrs	3rd Isolation 72Hrs
UBTH	4832(10)	+	++	++
UBTH	4832	+	++	++
UBTH	FBC2	+	++	++
UBTH	6B	-	-	+
UBTH	FBC	-	+	+

Note: + represents isolates that were positive
- represents isolates that were negative
- No visible change
+ Slightly dense
++ Dense
+++ Very dense

Plate 6: McCartney bottles containing isolates of *C. neoformans* on UBA (Urea Base Agar).

CHAPTER FOUR

DISCUSSION

The isolation of *C. neoformans* from pigeon droppings in the sites visited confirms pigeon as a habitat for this opportunistic pathogen and has been confirmed also by many researchers in different part of the world. Pigeon droppings have been reported as important substrate for the presence and maintenance of *C. neoformans* in the environment (Cassadevall et al., 1996). The presence of *C. neoformans* recovered in the environment is an important finding. This fungus has been reported as an agent of opportunistic infections such as meningitis, lung infections, fungemia, and abscess and skin infection mainly with patients with great deteriorations (Chang et al. 2004; Mitchel, and Perfect 1995).

In this study, all isolates were obtained from pigeon droppings, demonstrating that pigeon dropping is the prinicipal environmental sources for *C. neoformans*. Pigeon droppings have been known to be a good nutrient source for growth of *Cr. neoformans* (Chee and Kim, 2003). Especially, pigeon droppings in pigeon shelters had accumulated and remained there for a long time, and they possessed appropriate environmental conditions, such as darkness, humidity, and temperature, in which *Cr. neoformans* could survive and flourish. The occurrence of the agent of cryptococcosis in the areas of this study could be due to the environmental

conditions favoring 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 (Ruíz A, Formtling RA, Bulmer GS. 1981). Dry excrement is a favorable substratum since it has fewer bacteria and therefore less competition, which could help explain the higher population density found in this substratum (Ruíz A, Formtling RA, Bulmer GS. 1981). We added important information about the characteristics and pathogenicity of *C. neoformans*. Several factors including urease activity have been associated with the virulence of *C. neoformans* (Cox et al., 2000). Ability to grow at 37°C is generally accepted as virulence factor uniquely for *C. neoformans* (Kwon-Chung, et al., 1982; Kwon-Chung and Rhodes, 1986). Previous study showed that all isolates that were cultured and grown on plated SDA (Sabouraud Dextrose Agar) at 37C also came out positive on urease test using UBA (Urea Base Agar).

This study has attempted to match urease factor with that of temperature (ability to grow at 37°C) in screening and selection of pathogenic *C. neoformans.* The rapid detection of urease activity is one means of tentatively identifying *C. neoformans* from clinical specimens (Canteros et al.,1996) and (Zimmer and Roberts,1979). According to Casadevall and Steenberg (2003) urease enzyme plays a major role as a virulence factor of several pathogenic agents such as *Cryptococcus*

neoformans and Cryptococcus gatti. Kreger-Van-Rij (1984) studied urease activity for most of yeast species and she stated that, urease activity is one of the most important biochemical properties for members of the genus Cryptococcus and basidiomycetous yeast. Kwon-chung et al. (1987) reported that there are significant difference between two varites of *C. neoformans* especially with regards to the nature of their urease activity.

The results of the screening and selection showed that urease test can screen out samples that do not contain *C. Neoformans*. Interestingly, all the isolates that were positive at 37°C also tested positive for the urease test. This suggests that urease test can serve as complement alternative for screening and selection of pathogenic *C. neoformans.*

CONCLUSION

Cryptococcus neoformans was first described as pathogen at the end of the 19th century, although the characterization of its mechanisms has been mainly studied in the last four decades. Despite the great effort invested by the scientific community in this pathogen, there are still great challenges to manage and decrease its impact.

This experiment demonstrates the possibility of isolating *C. neoformans* at minimum temperature 37°C and urease activity tests. There are some liposomal formulations that reduce the negative effects of this antifungal organism, but their high prices difficult their application in developing countries. For these reasons, future research is still needed to understand the virulence of this fungal pathogen.

Also, people should be educated on all possible precaution or measures to be taken to prevent, reduce and control the spread of the anti-fungi disesase caused by this pathogenic organism. Pigeon sellers should be enlightened on how to maintain safety precautions in order for them not to be infected by this anti-fungi disease especially immunocompromised patients because it is observed that pigeon droppings are the main source of this pathogenic organism.

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