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Estimating the Frequency Distribution of Melanin Producing *Cryptococcus neoformans* In Benin City, Nigeria

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ABSTRACT

Cryptococcus neoformans, an opportunistic pathogenic fungus that causes severe infections like cryptococcosis, particularly in immunocompromised individuals such as HIV/AIDS patients. This research investigated the frequency distribution and prevalence of *C. neoformans* in three markets (Egor, New Benin and Uselu) in Benin City, Edo State, Nigeria. A total of 225 isolates sampled from bird droppings (as a known carrier of the pathogen) were tested for *C. neoformans* using urease and Niger seed agar. According to the sampling from the three markets, in 2023, Egor had 60 % positive isolates, while Uselu and new Benin had 58% each. In 2024, the prevalence increased to 84 % in Egor, 76 % in Uselu and 92 % in New Benin. These results showed that *C. neoformans* was present in all locations, with varying prevalence rates across 2 years. Thus, suggesting an overall increase in *C. neoformans* prevalence, posing a potential public health risk and hazard which may lead to death. This finding therefore, highlights the need for public awareness and preventive measures such as good hygiene and management practices, especially in market areas where pigeons are commonly found. One key virulence factor responsible for the distribution pattern and selection of *C. neoformans* is its ability to produce melanin(colour pigment) which contributes to the understanding of the pathogens ecology and epidemiology, thereby providing necessary information on disease management strategies. The results from the experiment indicates a potential danger in acquiring cryptococcosis and an intending need for public health interventions in mitigating the risk of *C. neoformans* infections.

INTRODUCTION

Cryptococcus neoformans, (a human pathogen) is a basidiomycetous yeast found in environments all over the world (Esakkiammal and Rajakumari, 2025). Pigeon guano, which is abundant in public areas, is known to be a potential carrier in the spread of this pathogenic yeast (Chee and Lee, 2005). C. neoformans is typically found in the waste surrounding pigeons nests, decaying wood, and soil polluted with pigeon or chicken droppings. *C. neoformans,* an opportunistic pathogenic fungus that causes severe infections like cryptococcosis, particularly in immunocompromised individuals such as HIV/AIDS patients.

Other species known as *C. gattii* can cause infections in are immunocompetent and healthy individuals. The incidence of *Cryptococcus* disease was 0.8 per million before the AIDS pandemic in the United States (Mirza *et al*., 2003), but in 1992, at the height of the epidemic, it rose to 5 per 100,000. However, the prevalence of *Cryptococcus* disease is quite high in sub-Saharan Africa, where the AIDS epidemic is raging. More people die from cryptococcal meningitis each year than from tuberculosis in sub-Saharan Africa, where it is the most prevalent culture positive meningitis.

The pathogenic fungus *Cryptococcus neoformans* can cause meningoencephalitis and other systemic disorders, as well as dangerous infections, especially in immunocompromised people. *C. neoformans* infection is more likely to occur in those with weakened immune systems, such as those with HIV/AIDS, organ transplant recipients, or those receiving chemotherapy (Zhao *et al.,* 2023). *C. neoformans* which is a causal agent of Cryptococcosis is identified by its ability to produce dark, melanin pigments with various phenolic compounds as substrates (Nurudeen and Ahearn, 1979). One of the primary virulence factors of this pathogen is its ability to produce melanin, a pigment essential for its survival and pathogenicity (Casadevall *et al.,* 2000). Since melanin is a critical virulence component that contributes to the survival and pathogenicity of *C. neoformans,* it is imperative to comprehend the distribution and selection for melanin synthesis in these populations in order to create efficient diagnosis and therapies (Kwon-Chung *et al.,* 2014).

The distribution pattern of melanin production in *C. neoformans* can provide valuable insights into the ecology and epidemiology of this pathogen as melanin is a key virulence factor, affecting survival in diverse environments and resistance to host defenses (Samarasinghe *et al.,* 2018). Recent studies shows that melanin production in *C. neoformans* isolates varies greatly, with some strains producing high levels of melanin while others produce little to none, highlighting the non-uniform distribution of this virulence factor (Chrissan *et al.,* 2020). This variation in melanin production may be influenced by environmental factors, such as temperature and pH, as well as genetic factors, such as mutations in the melanin biosynthesis pathway (Karkoszka *et al.,* 2024).

The primary research question is what the frequencies of distribution are and what key factors are responsible for distribution pattern and selection? The answers to these queries are centered on the objective of this work, which is to determine the frequency distribution of melanin production within a population of *C. neoformans* isolates collected from Benin City. The focus on distribution patterns and the selective pressures favouring melanin production of *C. neoformans*, ultimately contributes to the understanding of the pathogen's ecology, epidemiology, and informing disease management strategies.

MATERIALS AND METHODS

Sampling Site:

A total of 15 samples of pigeon droppings were collected (within the cages with pigeons) from three markets in Benin City (Egor, Uselu and New Benin market) which are located between latitude 6˚ 21' 33" N, Longitude 5˚36 ' 22" E, Latitude 6˚ 37 ' 42 " N and longitude 5˚ 61 ' 47 " E, latitude 6˚21' 3" N and longitude 5˚ 37 ' 53 "E respectively. 5 samples were collected from each market which are locatedas shown in the map below and *C. neoformans* were isolated from them. Sampling was carried out twice 2023-2024.

Isolation of Strains:

Isolation of strain was carried out following the method of Oghomwenakhin and Eboigbe (2019) with minor adjustments. In this work, instead of using Sabouraud dextrose agar (SDA), Yeast

peptone dextrose agar (YPDA) was used. About 1 g of pigeon dropping samples were added to 500 µL of Yeast Peptone Dextrose broth (YPD) in McCartney bottles, followed by mild shaking to allow the inoculum dissolve, then incubated at 25 ℃ for 24 hours. Approximately 100 µL Yeast Peptone Dextrose broth (YPD) was spread on Yeast Peptone Dextrose Agar (YPDA) medium and incubated using the Gallenhamp cooled incubator at 37 ℃ for a period of 5 days. Isolates from the cultures were further subcultured on YPDA to obtain contamination free single colonies. This process is to knockout every other organism in the sample gotten from the three locations. The resulting isolates were later cultured in Niger seed medium as a selective medium for *C. neoformans.*

Screening and Selections of Strains:

A total of 225 strains (150 strains in 2023 and 75 strains in 2024) isolated from the various samples were screened and *C. neoformans* were selected using urease test and Niger seed Agar for Confirmation test. Urease activity was carried out as described by Junior *et al.,* (2013): i.e. 20% urea solution was added to an already prepared Urea base agar medium. Afterwards, McCartney bottles with urease medium in slant were inoculated with *C. neoformans* and left at 25℃ for 48 hours. Observation was made for colour change. The initial colour of the medium was yellow before incubation at 25℃. A colour 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).

Niger seed agar was a selective medium used for the confirmation of *C. neoformans* in this work. The isolates were confirmed by their ability to produce melanin (dark pigmentation) during incubation for a minimum of 48 hours at a temperature of 37 ℃. 25 g of Niger seed (*Guizotia abyssinica*) was mashed and boiled for 30 minutes. 0.5 g of glucose, 0.5 g of potassium dihydrogen orthophosphate, 0.5 g of creatinine and 7.5 g of Agar were weighed and added to the infusion. The solution was thoroughly mixed before being diluted to 500 ryml with distilled water. The medium was sterilized and poured in petri dishes. After the medium solidifies, by serial dilution, the plates were inoculated with the same concentrations of inoculum for single colony isolation. The inoculated plates were then put into incubator to allow growth and production of melanin. The capability to grow at this temperature (37 ℃) was also used as a criterion for selection.

Distribution Pattern:

After the isolation and Confirmation test, the prevalence rate of *C. neoformans* was surveyed in the three market (Egor, New Benin and Uselu Market) to compare the distribution of *C. neoformans* in the bird droppings among the different locations.

RESULTS

The prevalence rate of *C. neoformans* in the various markets surveyed in Egor, Uselu and New Benin in 2023 is as shown in Figure 1 and Table 1. After the isolation process and urease test was carried out, the results showed that out of the total 150 isolates tested for *C. neoformans*, Egor had 30 out of 50 isolates, Uselu had 29 out of 50 isolates and New Benin had 29 out of 50 isolates that were positive to urease test. Egor had the highest prevalence percentage of *C. neoformans* compared to the other locations. This could be because this market had the highest number of bird droppings and the environment has conditions that favor the growth of *C. neoformans.*

Table 1: Environmental isolates that was positive and negative for *C. neoformans*

Site of Collection	Sample/Isolate	Presence of *C. neoformans*
Egor market	EG1	+
Egor market	EG2	+
Egor market	EG3	+
Egor market	EG4	+
Egor market	EG5	+
New Benin market	NB1	+
New Benin market	NB2	+
New Benin market	NB3	+
New Benin market	NB4	-
New Benin market	NB5	+
Uselu market	US1	+
Uselu market	US2	+
Uselu market	US3	+
Uselu market	US4	-
Uselu Market	US5	-

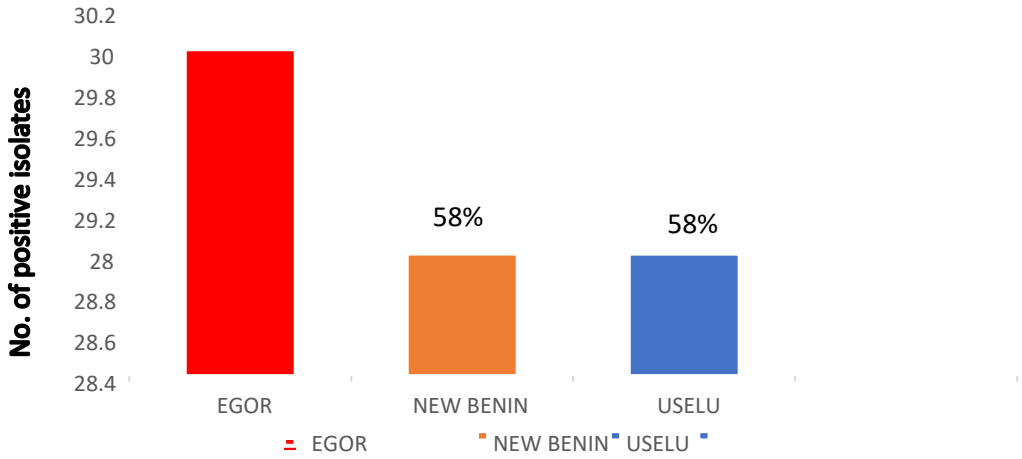

Fig. 1: The frequency distribution of *C. neoformans* in three markets in Benin City, Edo State, Nigeria, 2023.

The prevalence rate of *C. neoformans* in the various markets surveyed in Egor, Uselu and New Benin in 2024 is shown in Figure 2. The results showed that out of the 75 isolates tested for *C. neoformans,* Egor had 21 out of 25 isolates, Uselu had 19 out of 25 isolates and New Benin had 23 out of 25 isolates based on the urease test.

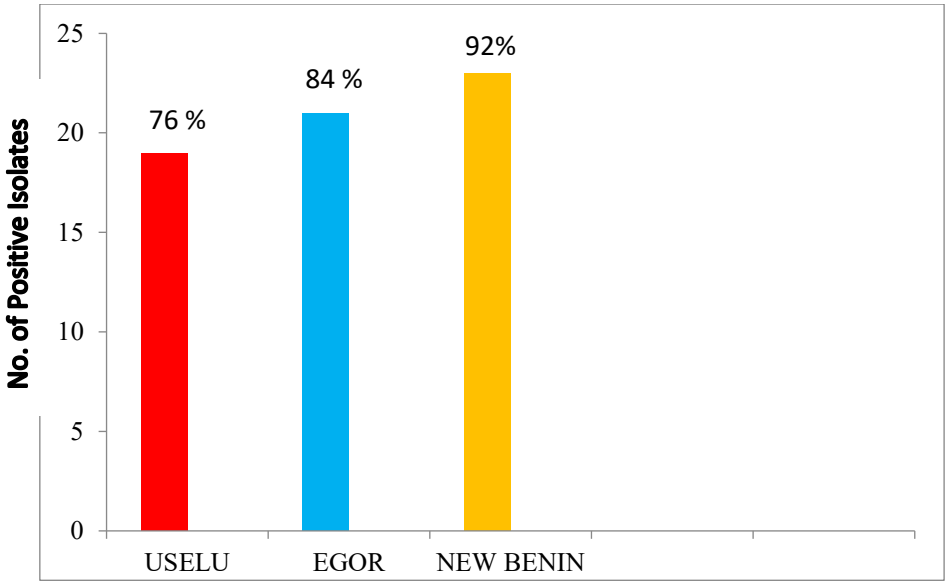

Fig. 2: The frequency distribution of *C. neoformans* in three markets in Benin City, Edo State, Nigeria in 2024.

DISCUSSION

Over the last twenty years, it is estimated that *Cryptococcus neoformans,* a human infectious disease organism, annually causes more than a million new cases of cryptococcosis and results in about 625,000 fatalities (Aguiar *et al.,* 2017; Ibe and Okoye, 2022). Pigeon droppings are regarded the primary environmental source of *C. neoformans* due to the high levels of urea and creatinine. Here, in this work samples of pigeon droppings were collected from three different markets in Benin City. After sampling, screening process was carried out and *C. neoformans* was identified in all the locations surveyed. Urease test and melanin production using Niger seed agar were routinely used to confirm the presence of *C. neofomans*.

Interestingly, *C. neoformans* was found to be present in all the areas sampled. Looking at the prevalence of *C. neoformans* in the various markets, the frequency distribution in the first sampling showed that *C. neoformans* were more prevalent in Egor market, while in the second sampling the *C. neoformans* became more prevalent in New Benin market. This suggests migration factor in the population (Amirrajab *et al*., 2016).

A critical analysis of the frequency distribution showed various levels of positivity rate for *C. neoformans*. In 2023, Egor has 60 % positive isolates while Uselu and New Benin market had 58 % each positive isolates. In 2024, Egor had 84 % positive isolates while Uselu had 76 % positive isolates, New Benin had the highest with 92 % positive isolates. In comparison, Egor increased from 60 % in 2023 to 84 % in 2024, a 24 % increase, while Uselu increased from 58 % in 2023 to 76 % in 2024, an 18 % increase. New Benin increased from 58 % in 2023 to 92 % in 2024, a 34 % increase. New Benin had the highest increase in prevalence percentage, while Egor also had high prevalence in both years. This increasing prevalence could be as a result of increase in human-animal contact (Del Poeta, 2012; Silva, *et al*., 2020). This work suggests an overall increase in *C. neoformans* prevalence across all locations, thus, posing a potential public health hazard due to human exposure to the airborne cells of *C. neoformans* in public areas. Since the people in these areas frequently observe feeding the pigeons and encouraging them to procreate, the findings of this study may indicate a danger for the acquisition of cryptococcosis (Kielstein *et al*., 2000; Olszewski, *et al.,* 2010; Ribeiro

et al., 2019; Esakkiammal and Rajakumari, 2025).

This research is serving as indirect estimate of the burden of cryptococcosis (Cryptococcal diseases). According to World Health Organization (WHO) developed Fungal Priority Pathogen List (FPPL), *C. neoformans* is a top-ranked fugal pathogen globally distributed that is primarily of environmental origin - pigeon droppings (Zhao *et al.,* 2023). On a global trend, the mortality rate of cryptococcosis is alarming, for this reason, we which to highlight the unawareness of individuals to the risk factors associated with this disease. Each year, an estimated 152,000 cases of cryptococcal disease occur among people living with HIV worldwide (Rajasingham *et al*., 2022). Among those cases, an estimated 112,000 deaths occur, the majority of which occur in Sub-Saharan Africa (Rajasingham *et al*., 2022). Here, we submit that many Nigerians are unaware of this pathogen causing life threatening diseases. This fact is drawn from our common experiences in the market in which there is no Hygiene in handling the pigeons. In addition to this limitation, there is no access to diagnosis and treatment of cryptococcosis similar to most fungal infection, due to lack of funds (Zhao *et al.,* 2023). This further heightens the threat of this disease if no adequate policy is established for the management of this disease.

CONCLUSION

In this research, *C. neoformans* was identified in all three areas of survey and confirmed for its virulence showing that there is a risk of *Cryptococcus* disease in those areas. The population survey of *C. neoformans* in Benin City provides insights on the prevalence, influencing factors and geographical spread of this fungal pathogen. This work emphasizes the significance of comprehending the mode of spread of this fungus and its implications in public health. Further research is necessary to develop specific measures in combating the spread of infection. Presently, the awareness about the health implication of the presence of *C. neoformans* in our market place is very low. Therefore, awareness programme is necessary in order to prevent the spread of diseases associated with this organism.

Declarations:

Ethical Approval: This study did not involve human or animal subject. Therefore, ethical consideration was not applicable.

Authors Contributions: Both authors contributed equally in the conceptualization and the design of the research protocol as well as participated in the laboratory work and writing of the manuscript.

Consent for publication: All authors agreed with the content and that all gave explicit consent to submit and that they obtained consent from the responsible authorities at the institute/organization where the work has been carried out, before the work is submitted.

Conflict of interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability Statement: All data used for the study are available in the manuscript and also upon request from the Principal Investigator.

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