

## ISOLATION OF *Cryptococcus neoformans* FROM ENVIRONMENTAL SAMPLES COLLECTED IN SOUTHEASTERN NIGERIA

Emeka I. NWEZE(1), Fred A. KECHIA(2,3), Uju E. DIBUA(1), Charles EZE(4) & Uwakwe S. ONOJA(5)

### SUMMARY

Cryptococcosis caused by *Cryptococcus neoformans* is the second most common fungal opportunistic pathogen and a life-threatening infection with serious clinical manifestations especially in HIV/AIDS and other immunocompromised patients. In Nigeria, HIV/AIDS infection has reached an alarming level. Despite this, information on the presence of this fungus in clinical and environmental samples is very scanty in Nigeria and many other parts of Africa. We set out to evaluate the presence of *Cryptococcus neoformans* or *C. gattii* in pigeon droppings obtained from Southeastern Nigeria. One hundred and seventy-seven samples of pigeon droppings from six sample types were collected. The area covered comprised of ten cities and other locations spanning across five States in Nigeria. Using established techniques, *Cryptococcus neoformans* was isolated from 39 of the 177 (22.0%) samples overall. No *C. gattii* was isolated. Most of the isolates (32.4%) were recovered from dovescotes (11 of 34) followed closely by samples taken from markets (31.8%; seven of 22) and least from the church (4.0%; one of 25). The highest isolation rate (38.9%) was found in samples from *Enugu-Ezike* (seven of 23) while the least came from *Afikpo* and the other locations each with 9.1% isolation rate. This is the first large-scale screening of *Cryptococcus neoformans* from pigeon droppings in Nigeria. The ecological and epidemiological significance of these findings are discussed.

**KEYWORDS:** *Cryptococcus neoformans*; Nigeria; Pigeon droppings; Isolation.

### INTRODUCTION

Infections by opportunistic mycoses including *Cryptococcus* have become a major clinical problem particularly in immunocompromised populations such as those living with AIDS<sup>12</sup>. *Cryptococcus neoformans* has been reported as the main microorganism that causes meningitis among adult HIV/AIDS patients in Africa and cryptococcal meningitis is now considered an AIDS-defining condition<sup>1,13,21</sup>. Information from WHO indicates that 34 million people suffer from HIV/AIDS, 2.7 million contract new HIV infections every year and 1.8 million die every year from AIDS-related causes. With this worrying and rapid increase in HIV/AIDS cases and the corresponding increase in the occurrence of cryptococcosis in this disease, the ecology of *Cryptococcus neoformans* has, undoubtedly, generated much interest and global attention. We previously reported that there was an alarming trend in HIV/AIDS infection in Nigeria, especially in the southern flank<sup>18</sup>. In fact, the greatest burden of cryptococcosis occurs in sub-Saharan Africa, where mortality is estimated to be 50% to 70%<sup>6,7</sup>.

There are two main etiological agents of cryptococcosis: *C. gattii* (serotypes B and C) and *C. neoformans* which is separated in two varieties, *var. grubii* (serotype A) and *var. neoformans* (serotype D).

Although all the serotypes might differ in their geographical spread, they can all cause disease in humans and animals. There is a report which suggests that *C. neoformans* is the most common *Cryptococcus* species affecting AIDS patients regardless of their geographical location<sup>11</sup>.

Environmental *C. neoformans* isolates were globally reported including samples from Africa. The main sources are pigeon and other birds' excreta, trunk hollows of different species of trees as well as bat guano. Most isolates have been recovered from hollows of living trees, pigeon droppings, bat guano, etc.

Pigeon rearing is common in Nigeria, especially in the southern flank and, in many cases, the pigeons are not bred indoors (kept in a protected cage) but outdoors as they are allowed to fly around the environment and return to their owners perhaps at dusk. This raises some health concerns, although the extent of these concerns is unknown to the best of our knowledge.

Although HIV/AIDS epidemic has an alarming trend in southern Nigeria, there are not many reports on the frequency and significance of *Cryptococcus neoformans* in pigeon droppings with only a single report published from samples taken from one city<sup>14</sup>.

(1) Department of Microbiology, University of Nigeria, Nsukka, Nigeria.

(2) Department of Biomedical Sciences, Fac. of Health Sciences, University of Bamenda, Cameroon.

(3) Medical/Clinical Mycology, Faculty of Medicine and Biomedical Sciences, University of Yaounde I, Cameroon.

(4) Department of Medical Biochemistry, University of Nigeria, Enugu, Enugu State Nigeria.

(5) Department of Human Nutrition and Dietetics, University of Nigeria, Nsukka, Enugu State, Nigeria.

**Correspondence to:** Nweze Emeka I., PhD, AIMLS, Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria, Phone: +2348068535841. Email: nwezemekai@yahoo.com

In this study, we carried out an expanded investigation in southeastern Nigeria involving 10 different geographical locations in order to obtain a more robust knowledge of the occurrence of *Cryptococcus neoformans* in pigeon droppings and ascertain the relevance of this important source of infection in an area with a worrying escalation in HIV/AIDS scourge.

## MATERIALS AND METHODS

**Geographical/study location:** One hundred and seventy-seven dried samples of pigeon droppings were collected from ten major locations and other sites in southeastern Nigeria (Fig. 1) to investigate the ecological and epidemiological relevance of pigeon droppings in cryptococcosis over a period of eight months. Samples were collected in sterile plastic bags from roofs of private and public buildings and other places inhabited by pigeons such as dovecotes, places of worship (churches), markets, motor parks and uncompleted buildings. The collected samples were properly packaged and stored at room temperature until the isolation was performed.

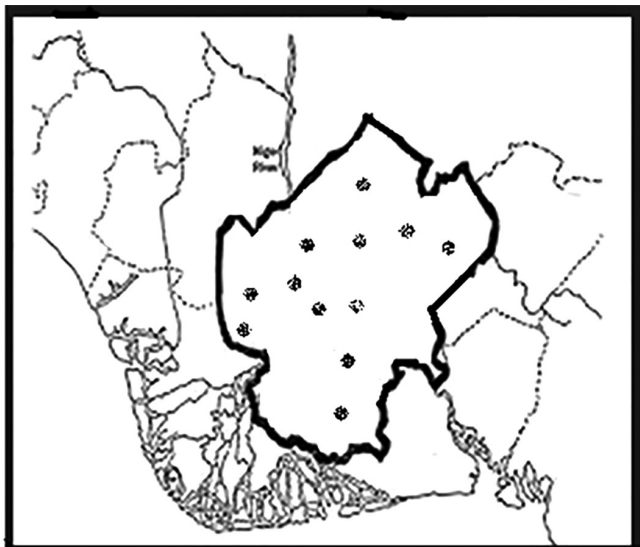


Fig. 1 - Map of Nigeria showing the area (center map) covered by the study. The dots represent locations where collected samples tested positive for *Cryptococcus neoformans*.

**Isolation and identification of the *Cryptococcus* strains:** First, about 1 g of each sample was suspended in 20 mL of sterile saline solution. The saline solution/pigeon dropping mixture was allowed to settle for 35 minutes after vortexing for three minutes. Next, about 60  $\mu$ L of each sample was inoculated on Niger seed agar (Staib agar medium) containing chloramphenicol and biphenyl at a concentration of 0.5 g/L and 100 mg/L, respectively and dispensed in Petri dishes. The inoculated plates were incubated for 14 days at 26 °C but were monitored daily from day 2 to observe brown pigmented colonies that were temporary identified as *Cryptococcus neoformans* species complex. Subsequent and final identification of isolates involved cyclohexamide sensitivity, myo-inositol assimilation, thermotolerance at 37 °C and urease production. For determining the species of the isolates, Canavanine-glycine-bromothymol blue agar medium (CGB) was used by streaking each isolate on the medium and observing color changes after two to five days incubation at 26 °C.

Differentiation of *var. grubii* (serotype A) from *var. neoformans* (serotype D) was performed using a method previously described<sup>8</sup>. Two *C. neoformans* isolates H99 (serotype A) and NIH B4476 (serotype D) were used as control strains.

## RESULTS

Thirty nine out of the 177 samples of pigeon droppings screened from six different sample types were positive for *C. neoformans* occurring in a percentage of 22.0%. Eleven isolates (32.4%) were recovered from dovecotes (structures housing pigeons), nine (19.1%) were recovered from samples collected from roofs of buildings, while eight (24.2%) were recovered from samples collected from the interior of uncompleted buildings. The remaining positive samples were recovered from a market (seven, 31.8%), motor parks (three, 18.8%) and a church (one, 14.0%) (Table 1). Positive samples were recovered from nine major cities in southeastern Nigeria except for three isolates. These three isolates were recovered from 33 samples randomly collected from smaller villages and communities in the southeast of the country. Of note, however, is the fact that only three samples out of a total of 33 taken from two of the 10 communities/smaller towns were positive: *Obollo-Afor* (two) and *Orba* (one). They are both located in *Udenu* Local Government areas in the *Enugu* State. The map of Nigeria showing the area covered by our study is shown in Figure 1. The isolation rates for each of the ten major cities/towns where samples were collected ranged from 9.1% (one out of 11 and three out of 33) to 38.9% (seven out of 23) with *Enugu-Ezike* having the highest rate (Table 2). All isolates were found to be *C. neoformans* and none were urease-negative. When data were compared among the five States comprising the southeast flank, *Enugu* State appeared to record the highest isolation rate (data not shown). All the *C. neoformans* isolates recovered belonged to the *var. neoformans*.

Table 1  
Sample sources and isolation rates of *Cryptococcus neoformans*

Source	No. of samples	No. positive	Percentage
Roof of buildings	47	9	19.1
Motor parks	16	3	18.8
Church	25	1	4.0
Dovecotes	34	11	32.4
Market	22	7	31.8
Uncompleted buildings	33	8	24.2
<b>Total</b>	<b>177</b>	<b>39</b>	<b>22.0</b>

## DISCUSSION

Recent available information revealed that of the 19,753 *C. neoformans* and *C. gattii* strains so far reported from 25 out of 58 African countries, 79% alone were from South Africa. Furthermore, environmental surveys performed in eight countries namely Nigeria, Egypt, Tunisia, Democratic Republic of Congo, Zimbabwe, Burundi, South Africa and Botswana recovered only 1% of the total isolates reported so far<sup>4</sup>. This among other data shows that environmental isolation of *C. neoformans* and *C. gattii* is grossly underreported in Africa and

**Table 2**

Samples origin and distribution of *C. neoformans* from pigeon droppings in southeastern Nigeria

Location/State	Total No. of samples	Total No. of positive samples (%)
Abakiliki/Ebonyi	13	3 (23.1)
Afikpo/Ebonyi	11	1 (9.1)
Awka/Anambra	11	2 (18.2)
Enugu-Ezike/Enugu	23	7 (38.9)
Enugu & 9 <sup>th</sup> mile Corner/Enugu	14	6 (30.4)
Nsukka/Enugu	24	6 (25.0)
Oji-River/Enugu	10	2 (20.0)
Onitsha/Anambra	17	5 (29.4)
Owerri/Imo	10	2 (20.0)
Umuahia/Abia	11	2 (18.2)
Others	33	3 (9.1)
	177	39 (22.0)

this was one of the major reasons for our study. Available data indicate that increasing numbers of *Cryptococcus* infection have been reported especially in AIDS patients. However, only a few cases have been reported in Nigeria despite the increasing number of AIDS cases<sup>18</sup>.

Currently, there is scanty information on the isolation of *Cryptococcus neoformans* from pigeon droppings in Nigeria. To the best of our knowledge, the only study reported on the relevance of pigeon droppings in cryptococcosis was confined to a single city<sup>14</sup>. Our main aim was to expand the study in a larger scale and cover as many parts of southern Nigeria as possible. However, the difficulty in sample collection limited our coverage to ten cities comprising five States in Nigeria. Despite this, our study is the first large-scale research in Nigeria demonstrating the isolation of this pathogenic fungus from pigeon droppings.

Our data showed an isolation rate of 22.0% (39 out of 177). This is higher than the 14.4% reported previously in *Awka*<sup>14</sup> (one of the cities included in our study). We found an isolation rate of 18.2% in this same city. Unlike ours, the report from *Awka* did not investigate the species and variety of the isolates recovered. A much older, but not closely related, study isolated *Cryptococcus* from the feces of captive birds in a wildlife park located in *Jos*, northern Nigeria<sup>9</sup>. This study cannot be compared with ours. Similar studies have been conducted in other African countries<sup>16,19</sup> and even in other continents<sup>5-10</sup> but we observed that unlike our study, many of those were carried out within one specific location. These studies reported an isolation rate that varied from 4.0 to 87.5%. We are not sure why the isolation rate varies from one location to another. One possible reason might be due to the prevailing conditions peculiar to each location. We were not able to isolate *C.gattii* in our study. The reason for this is unknown but it has been observed that although *C. neoformans/C. gattii* ratio is variable for each continent, it is often higher in favor of *C. neoformans*. For example, it is 88:1 in Africa, 68:1 in Europe, 7.6:1 in Asia, 4.5:1 in Central and South America and 3.5:1 in North America<sup>4</sup>.

Clinically, our findings are relevant in many ways, especially owing to the fact that *C. neoformans* has been widely documented as

an opportunistic pathogen with predilection to the meninges and lungs. It also causes abscesses, fungemia and skin infections, especially in severely immunocompromised subjects<sup>3,15</sup> such as HIV/AIDS patients.

The increasing number of cases of HIV/AIDS observed in a steady basis in Nigeria means that many more of these infected patients will certainly attain the immunocompromised status over time and face challenges arising from opportunistic infections of which *Cryptococcus neoformans* has become a prominent player. This is possible because of the apparent lack of adequate HIV/AIDS prevention and control strategies in Nigeria. For example, antiretroviral therapy is not readily and widely available to all HIV/AIDS patients who need it and, even worse, certain uneducated/non-enlightened HIV/AIDS infected people refuse to admit that HIV/AIDS is real and therefore continue to harbor the virus without treatment and could potentially infect others with the virus. Therefore, the exposure to isolates of *C. neoformans* could likely be associated with infection risk in any given population<sup>2</sup>. In a previously published investigation, one of us already showed that HIV/AIDS has reached an alarming proportion in Nigeria, especially in the southern flank<sup>18</sup>.

In the current study, the isolation rate varied from city to city but there was no specific clear-cut trend in terms of the frequency of isolation from pigeon droppings in the different cities/towns investigated. However, the highest frequency (38.9%) of isolations from pigeon droppings was obtained from *Enugu-Ezike*. This is possibly due to the rearing of pigeons which is common among the inhabitants of this area. In the 9<sup>th</sup> mile corner of *Enugu* city the next high frequency of isolation (30.4%) was found. In this case, pigeon rearing is not a major practice in these areas but we observed several stray pigeons especially in the surrounding suburbs.

In literature, there are reports showing that pigeon droppings contain important substrates for the survival and maintenance of *C. neoformans* in the environment in addition to other sources<sup>20,22</sup>. We believe that the environmental conditions in eastern Nigeria are suitable for the survival of *C. neoformans*. The mostly dry excreta collected in all study locations have been previously reported to favor the growth of the fungus perhaps due to the reduced population of bacteria which would have competed with the fungus for nutrients. The ambient temperature and pH conditions in the region also favor this opportunistic pathogen.

A possible reason for the presence of the fungus in almost all the locations where samples were collected could be the migratory nature of the pigeons associated with the fact that many people who rear these birds do not confine them (keep them indoors). For instance, it is possible that pigeons in the 9<sup>th</sup> mile corner could fly over to *Enugu* city which is about a 15 minutes' drive away. This proximity even necessitated the grouping of 9<sup>th</sup> mile corner and *Enugu* together in this study. It is disturbing to note that *Cryptococcus neoformans* was recovered from some samples collected from public places such as motor parks, markets and roofs of public and private buildings such as school, hospital, and offices, etc. The reduced frequency of the fungus in certain locations could be due to a lower number of pigeons in such areas.

In conclusion, the education of residents especially those affected by HIV/AIDS on the ecological and epidemiological relevance of pigeons droppings in the environment is needed. Furthermore, adequate and necessary precautions should be taken by relevant authorities to limit human infection by *Cryptococcus neoformans* via pigeon droppings.

## RESUMO

### Isolamento de *Cryptococcus neoformans* de amostras ambientais coletadas no sudeste da Nigéria

A criptococose, causada por *Cryptococcus neoformans*, é o segundo patógeno fúngico oportunista mais comum em infecções com risco de vida e manifestações clínicas graves, especialmente em HIV/AIDS e outros pacientes imunocomprometidos. Na Nigéria, a infecção pelo HIV/AIDS atingiu um nível alarmante. Apesar disso, informações sobre a presença desses fungos em amostras clínicas e ambientais é muito escassa na Nigéria e em muitas outras partes da África. Propusemo-nos a avaliar a presença de *Cryptococcus neoformans* ou *C. gattii* em fezes de pombos obtidos do sudeste da Nigéria. Foram coletadas 177 amostras de fezes de pombos de seis localidades. A área coberta foi composta por dez cidades e outras localidades abrangendo cinco Estados na Nigéria. Usando técnicas estabelecidas, *Cryptococcus neoformans* foi isolado de 39 do total de 177 (22,0%) amostras. Nenhuma amostra de *C. gattii* foi isolada. A maioria dos isolados (32,4%) foi recuperada de pombais (11 de 34), seguido de perto por amostras colhidas em mercados (31,8%; 7 de 22) e por último na igreja (4,0%; 1 de 25). A maior taxa de isolamento (38,9%) foi encontrada em amostras de Enugu-Ezike (7 de 23), enquanto a menor taxa foi de Afikpo e os outros locais, cada um deles com taxa de isolamento de 9,1%. Esta é a primeira triagem em larga escala de *Cryptococcus neoformans* em fezes de pombos na Nigéria. A importância ecológica e epidemiológica destes achados é discutida.

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