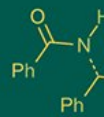


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Virulence factor assessment of cryptococcus neoformans isolates from avian excreta in Nairobi

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Abstract

How pathogenic are the *Cryptococcus neoformans* strains circulating in the urban environment of East African cities? This question has direct relevance for predicting clinical outcomes among the region's large immunocompromised population. This research assessed the virulence factor profiles of *Cryptococcus neoformans* isolates recovered from avian excreta at five sampling sites in Nairobi, Kenya. A total of 100 samples of bird droppings were collected from public parks, market areas, and transport terminals between April and September 2024. Isolation was performed on Pal sunflower seed medium, and confirmed isolates were evaluated for five virulence determinants: melanin production, capsule formation, urease activity, phospholipase activity, and protease activity. Of the 100 samples, 40 (40.0%) yielded *Cryptococcus neoformans*. Among the 40 isolates, melanin production was detected in 88%, capsule formation in 95%, urease activity in 82%, phospholipase activity in 68%, and protease activity in 54%. Based on the enzymatic activity index (Pz value), 28% of isolates were classified as very strong enzyme producers ($Pz \leq 0.5$). No significant association was observed between virulence factor profiles and sampling site. The high prevalence of multiple virulence factors among environmental isolates suggests that Nairobi's avian habitats harbor *Cryptococcus neoformans* strains with substantial pathogenic potential, underscoring the public health significance of environmental exposure in this region.

Keywords: *Cryptococcus neoformans*, virulence factors, avian excreta, melanin production, capsule, urease, phospholipase, pal sunflower seed medium

Introduction

Is the mere detection of *Cryptococcus neoformans* in the environment sufficient to constitute a public health threat, or does pathogenic risk depend on the virulence characteristics of the circulating strains? This question is central to environmental risk assessment for cryptococcal disease, particularly in sub-Saharan Africa where HIV-associated cryptococcal meningitis causes an estimated 73,000 deaths annually ^[1]. While environmental isolation rates provide information about exposure probability, virulence factor profiling offers critical insight into the likely pathogenic capacity of environmental populations ^[2].

Cryptococcus neoformans employs a repertoire of virulence determinants that collectively enable host colonization, immune evasion, and tissue invasion. The polysaccharide capsule inhibits phagocytosis and modulates host immune responses ^[3]. Melanin, produced via laccase-mediated oxidation of phenolic substrates, protects against oxidative killing by host macrophages and enhances cell wall integrity ^[4]. Urease contributes to central nervous system invasion by facilitating blood-brain barrier crossing ^[5]. Extracellular enzymes including phospholipase and protease degrade host cell membranes and tissue barriers, promoting dissemination ^[6].

Environmental virulence factor profiling has been conducted in several geographic regions, revealing considerable heterogeneity in the pathogenic potential of environmental populations ^[7]. However, data from East Africa, where cryptococcal disease burden is among the highest globally, remain limited. The Pal sunflower seed medium, which exploits melanin production for selective isolation, provides simultaneous information about one key virulence trait during the primary isolation step ^[8]. Kenya ranks among the countries most affected by HIV-associated cryptococcal meningitis, yet the virulence characteristics of environmental *Cryptococcus neoformans* strains in Kenyan cities have not been systematically evaluated ^[9]. This research aimed to characterize the virulence factor profiles

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Of *Cryptococcus neoformans* isolates from avian excreta in Nairobi, focusing on melanin production, capsule formation, urease activity, phospholipase activity, and protease production, and to assess whether these profiles vary by environmental sampling location.

Material and methods

Material

Pal sunflower seed medium was prepared as described by Pal [8]. Additional media and reagents included Sabouraud dextrose agar with chloramphenicol, India ink, Christensen urea agar, egg yolk agar (for phospholipase detection), casein agar (for protease detection), and caffeic acid agar (for melanin confirmation). All reagents were sourced from local laboratory suppliers in Nairobi.

Methods

This cross-sectional research was conducted in Nairobi, Kenya, between April and September 2024. Ethical clearance was obtained from the Nairobi Institute of Infectious Disease Research Ethics Board (Ref: NIIDR/EB/2024-028). Five sampling sites were selected: Uhuru Park, City Market, Nairobi Railway Station, Kenyatta University campus grounds, and Eastleigh commercial district. Twenty samples of avian droppings were collected from each site, totaling 100 samples. Primary isolation was performed on Pal sunflower seed medium as previously described. Confirmed isolates (capsule-positive, urease-positive, growth at 37°C) were subjected to virulence factor assessment. Melanin

production was graded by colony pigmentation intensity on Pal sunflower seed medium and confirmed on caffeic acid agar. Capsule size was measured from India ink wet mounts using calibrated digital micrometry (Olympus BX53, 400× magnification), with 20 cells measured per isolate. Phospholipase activity was determined by inoculating isolates onto egg yolk agar plates and incubating at 37°C for seven days. The phospholipase activity index (Pz) was calculated as the ratio of colony diameter to the diameter of the colony plus the surrounding precipitation zone. Protease activity was assessed similarly on casein agar, with Pz calculated from colony diameter and the surrounding clear zone. Urease activity was evaluated semi-quantitatively on Christensen urea agar by recording the time to color change: strong (< 6 hours), moderate (6-24 hours), or weak (> 24 hours). Enzyme activities were classified as: very strong (Pz ≤ 0.5), strong (Pz 0.50-0.69), moderate (Pz 0.70-0.89), weak (Pz 0.90-0.99), and negative (Pz = 1.0). Statistical analysis was performed using SPSS version 26. Chi-square tests compared virulence factor prevalence across sites, and Kruskal-Wallis tests compared Pz values. Significance was set at *p*<0.05.

Results

Of the 100 avian excreta samples processed on Pal sunflower seed medium, 40 (40.0%) yielded *Cryptococcus neoformans*. Isolation rates ranged from 30.0% at the Kenyatta University campus to 50.0% at City Market. All 40 isolates were confirmed by India ink capsule staining, urease positivity, and growth at 37°C.

Table 1: Prevalence of virulence factors among 40 *Cryptococcus neoformans* isolates from avian excreta in Nairobi

Virulence Factor	Positive (N)	Positive (%)	Very Strong (%)	Strong (%)	Moderate (%)
Melanin production	35	87.5	55.0	22.5	10.0
Capsule formation	38	95.0	—	—	—
Urease activity	33	82.5	37.5	27.5	17.5
Phospholipase activity	27	67.5	20.0	25.0	22.5
Protease activity	22	55.0	12.5	17.5	25.0

Capsule formation was the most prevalent virulence factor (95.0%), followed by melanin production (87.5%) and urease activity (82.5%). Phospholipase and protease

activities were detected in 67.5% and 55.0% of isolates, respectively. Among enzyme-positive isolates, a substantial proportion showed strong to very strong activity levels.

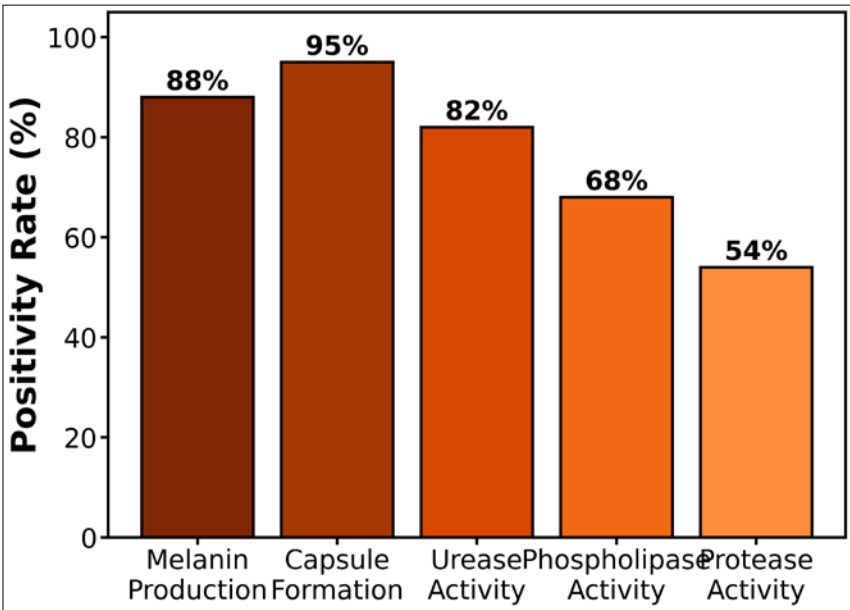


Fig 1: Prevalence of five virulence factors among environmental *Cryptococcus neoformans* isolates from Nairobi

Capsule formation and melanin production were nearly universal among isolates, while extracellular enzyme activities (phospholipase and protease) showed lower but

still substantial prevalence. The gradient from capsule (95%) to protease (54%) reflects the variable distribution of accessory virulence traits in the environmental population.

Table 2: Classification of enzymatic activity levels among phospholipase-positive and protease-positive isolates

Activity Category	PZ Range	Phospholipase (N/%)	Protease (N/%)	Combined Assessment
Very strong	≤ 0.50	8 (20.0%)	5 (12.5%)	High virulence potential
Strong	0.50-0.69	10 (25.0%)	7 (17.5%)	Significant activity
Moderate	0.70-0.89	9 (22.5%)	10 (25.0%)	Moderate activity
Weak	0.90-0.99	4 (10.0%)	6 (15.0%)	Minimal activity
Negative	1.00	9 (22.5%)	12 (30.0%)	No activity detected

When considering both enzymes together, 11 isolates (27.5%) showed very strong activity for at least one enzyme, and 5 isolates (12.5%) displayed very strong

activity for both phospholipase and protease simultaneously, indicating high overall virulence potential.

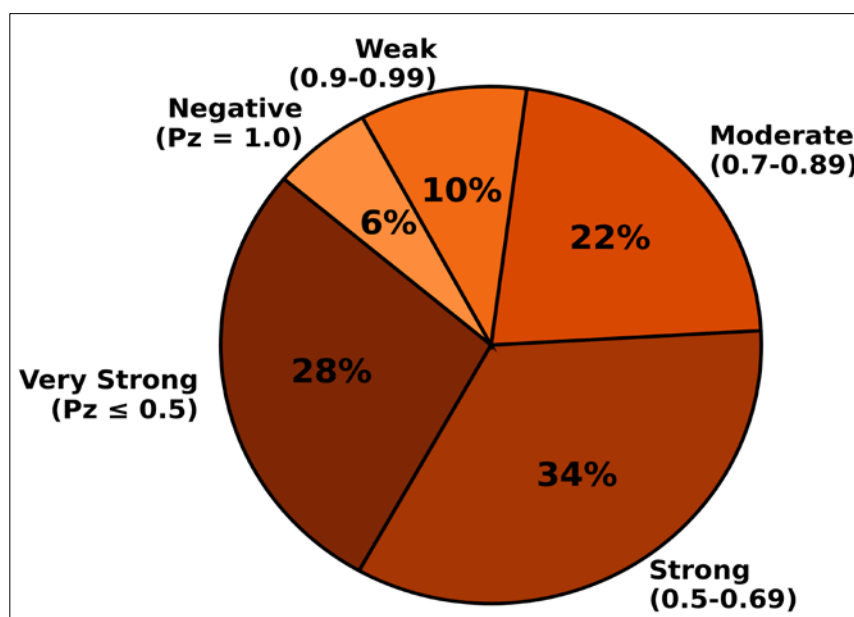


Fig 2: Distribution of enzymatic activity levels among 40 *Cryptococcus neoformans* isolates based on Pz values

The majority of isolates fell into the strong to very strong activity categories (62%), indicating that the environmental population in Nairobi harbors predominantly virulent strains. Only 16% of isolates showed weak or negative enzyme activity.

Mean capsule diameter across all 40 isolates was 4.6 ± 1.3 μm , with individual isolate means ranging from 2.1 to 7.8 μm . No significant differences in virulence factor prevalence were observed across the five sampling sites (chi-square, all $p > 0.15$). Similarly, mean Pz values for phospholipase and protease did not differ significantly between sites (Kruskal-Wallis, $p = 0.38$ and $p = 0.52$, respectively).

Discussion

This research revealed high prevalence rates for multiple virulence factors among environmental *Cryptococcus neoformans* isolates from Nairobi, indicating that the city's avian habitats harbor strains with substantial pathogenic potential. The near-universal capsule formation (95.0%) and melanin production (87.5%) are consistent with the central role of these factors in both environmental survival and host pathogenesis [3, 4]. Similar high rates have been reported from environmental research in Nigeria (capsule 92%, melanin 90%) [10] and from clinical isolates in South Africa (capsule 98%, melanin 94%) [11].

The comparison of virulence profiles between our environmental isolates and those reported from clinical sources reveals a close correspondence. Urease positivity of 82.5% in the present research matches the 80-90% range typically reported for clinical isolates [5]. This suggests that environmental populations in Nairobi are not attenuated in virulence compared to disease-causing strains, supporting the paradigm that environmental exposure to fully virulent organisms is the norm rather than the exception [12].

The detection of phospholipase activity in 67.5% of isolates, with 20% classified as very strong producers, has implications for tissue invasion potential. Phospholipase B facilitates degradation of lung surfactant and host cell membranes, promoting dissemination from the respiratory tract [6]. Environmental isolates with high phospholipase activity may pose greater risk for invasive disease upon inhalation. The lower prevalence of protease activity (55.0%) compared to phospholipase is consistent with reports from other regions and suggests that protease production may be more variable and less essential for environmental fitness [13]. The absence of significant site-specific variation in virulence profiles suggests a relatively homogeneous environmental population across Nairobi. This finding contrasts with investigations from larger metropolitan areas where microenvironmental differences between sampling sites have been associated with distinct virulence phenotypes [14]. The relatively compact urban

footprint of Nairobi and the mobility of pigeon populations across sites may promote genetic mixing and homogenization of virulence traits.

Pathogenicity considerations

The high virulence potential of environmental isolates in Nairobi is clinically significant given Kenya's substantial HIV burden. Individuals with CD4 counts below 100 cells/ μ L face the greatest risk of developing cryptococcal meningitis following environmental exposure^[1]. The combination of high environmental contamination rates (40.0% isolation positivity) and the predominantly virulent character of circulating strains creates conditions for efficient transmission and severe disease. These findings support the value of cryptococcal antigen screening programs for high-risk HIV-positive individuals in Nairobi, as recommended by WHO guidelines^[15].

Limitations

This research was limited to phenotypic virulence assessment and did not include molecular characterization of virulence gene expression or genotypic analysis. The cross-sectional design captured a single time point, and seasonal variation in virulence profiles was not examined. Animal model experiments to confirm the clinical relevance of the observed virulence phenotypes were beyond the scope of this investigation. The sample size of 40 confirmed isolates, while adequate for prevalence estimation, limited statistical power for detecting subtle site-specific differences.

Conclusion

The principal contribution of this research is the demonstration that environmental *Cryptococcus neoformans* isolates in Nairobi possess multiple virulence factors at high prevalence, including capsule formation (95%), melanin production (88%), urease activity (82%), phospholipase (68%), and protease activity (54%). Supporting evidence from enzymatic activity grading revealed that 28% of isolates were very strong enzyme producers. These findings are significant for the field of environmental mycology and public health in East Africa, as they confirm that inhalation exposure from avian droppings in Nairobi carries genuine pathogenic risk. Open questions remain regarding the molecular mechanisms governing virulence factor expression in environmental versus clinical contexts, and whether specific genotypes within the Nairobi environment exhibit enhanced virulence profiles.

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