

Isolation of *Cryptococcus neoformans* from droppings of the Feral Pigeon (*Columba livia*) found in public places in El Salvador

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Abstract

Aim: The objective of this study was to isolate yeasts belonging to the *Cryptococcus neoformans* species complex from the feral pigeon (*Columba livia*) droppings collected from public squares and parks in El Salvador.

Methods: Samples were seeded in conventional culture media and confirmed colonies were typed by the enzymatic restriction technique of the URA5 gene.

Results: Of a total of 66 samples analyzed, three were positive for yeasts belonging to the *Cryptococcus neoformans* species complex. The molecular study grouped the isolates into molecular types VNI and VNII; both belong to the *Cryptococcus neoformans sensu stricto* species.

Conclusions: In the sites studied, the presence of this yeast is very low, probably due to environmental factors. The first report of *Cryptococcus neoformans sensu stricto* genotypes VNI and VNII in El Salvador is presented. This species is of public health relevance because it is responsible for more than 90% of cryptococcosis cases worldwide.

Keywords: *Cryptococcus neoformans*, *Columba livia*, genotype, zoonosis, public health.

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Pigeons have generally been considered a symbol of peace. The Feral pigeon (*Columba livia*) is considered a harmless animal and one of those that have the most contact with humans, however, they are potential transmitters of diseases and cause the deterioration of monuments and buildings in cities around the world.^{1,2,3} This pigeon species carries 60 different pathogenic organisms, five of which are regularly transmitted to humans. Among these five, the *Cryptococcus neoformans* species complex (consisting of *C. neoformans sensu stricto*, formerly known as *C. neoformans var. grubii*, and *C. neoformans*, formerly *C. neoformans var. neoformans*) ranks second in frequency.^{2,4,5}

This pathogenic yeast causes cryptococcosis, an opportunistic mycosis whose most notorious symptoms for the meningeal form are headache and visual disturbances. Since cryptococcosis usually manifests in immunodeficient individuals, it is considered a disease associated with Acquired Immune Deficiency Syndrome (AIDS) and testing for Human Immunodeficiency Virus (HIV) should be indicated whenever a species belonging to the *C. neoformans* species complex is isolated from clinical specimens.^{6,7} In some patients, certain conditions (such as

cryptococcaemia and high intracranial pressure) increase the likelihood of cryptococcosis being fatal.⁷ In El Salvador, more than 500 cases of cryptococcosis were reported between 2009 and 2019; of these, 43 were fatal cases (Ministry of Health of El Salvador).

Although the sites of contagion and the link of this pigeon species to cases such as these are unknown, public places frequented by them could be potential foci of contagion. Therefore, given that the public places visited in this study are frequented by many people for various activities, the risk of exposure could represent a health hazard for people, especially immunodeficient people. The objective of the study was to isolate yeasts belonging to the *C. neoformans* species complex from the droppings of Feral pigeons (*C. livia*), using samples collected in a selection of parks and public squares in El Salvador.

Methods

Sample collection: This research was observational and qualitative; study sites were chosen at convenience. The collection of fecal samples of Feral pigeons (*C. livia*), was performed in places with a large influx of people and pigeons (atrium of cathedrals, squares, and public parks) throughout the eight most densely populated departments of El Salvador, according to the last population census conducted by the General Directorate of Statistics and Census in 2008, which are: San Salvador, La Libertad, Santa Ana, Sonsonate, San Miguel, Usulután, La Paz and Cuscatlán (Figure 1).

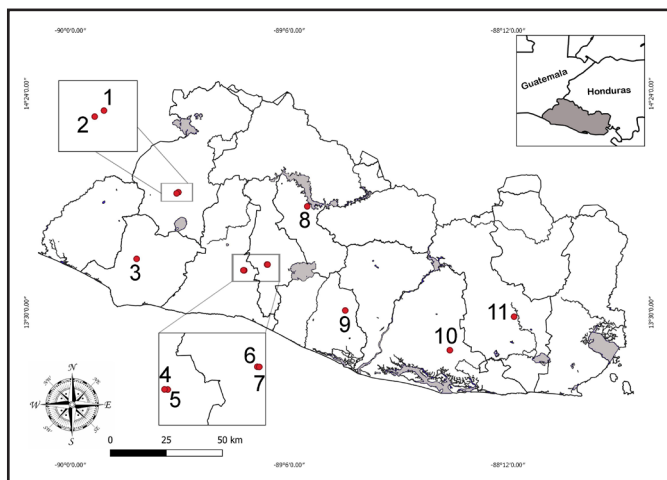


Figure 1. Location of parks and public squares visited for the collection of the samples of droppings of the Feral pigeon (*Columba livia*). 1) Santa Ana Cathedral, 2) El Calvario Park, 3) Rafael Campo Park, 4) San Martín Park, 5) Daniel Hernández Park, 6) Gerardo Barrios Square, 7) Libertad Square, 8) Suchitoto Central Park, 9) Zacatecoluca Civic Square, 10) Usulután Central Park and 11) Guzmán Park.

Droppings were collected whenever there was effective verification that they belonged to Feral pigeons (*C. livia*). Aspects such as the morphology of the droppings of this species were considered to avoid collecting fecal material of another species. Each study site was visited during the morning, from 7:00 am to 12:00 noon. Small sterile plastic spatulas were used, one for each sample collected. Two excrement samples were taken per site. Three collection trips were made to each site every two weeks. All samples obtained by spatula collection were placed in sterile Ziploc® plastic bags and transported in a dry plastic container the same day or the day after collection to the laboratories of the Centro de Investigaciones y Desarrollo en Salud located in the facilities of the Universidad de El Salvador for processing.

Isolation of the *C. neoformans* complex: To isolate and identify the *C. neoformans* complex, the methodology detailed in a previous study in El Salvador was followed.⁸ First, the excreta were placed in a flask with 30 mL of 0.85% saline solution. This was then placed in a magnetic stirrer, the contents were stirred for 10 minutes and allowed to settle. Subsequently, 1.0 mL of the supernatant was taken and diluted in another flask containing 9 mL of 0.85% saline solution. 0.1 mL of the resulting 10 mL was taken and placed on Petri dishes with expanded Sunflower Seed Agar with a Driglaski spatula; this is a selective and differential culture medium, simple to prepare, and inexpensive.⁹ It was then incubated at 37 °C for 3 to 7 days.

Samples were taken from the brown circular colonies that grew on Sunflower Seed Agar and seeded in test tubes containing Sabouraud's Glucose Agar in bevels using the loop streaking technique and incubated at 28°C. Suspect colonies were sent to the Medical Mycology Section of the Faculty of Microbiology of the University of Costa Rica for confirmation of the species. To prepare the isolates to be sent, a sample of the suspicious colonies was taken on Sabouraud Glucosated Agar and placed in a 1.5 mL conical tube with 1.0 mL of sterile 0.85% saline solution and properly labeled.

Phenotypic characterization of *Cryptococcus* isolates: At the Medical Mycology Section of the University of Costa Rica, a series of phenotypic tests were subjected to a series of phenotypic tests to verify their purity and correct classification within the *C. neoformans* complex. They were negatively stained with India ink, and inoculated on Christensen's Urea Agar, L-Dopa

agar, and canavanine-glycine-bromothymol blue agar (CGB). In addition, a biochemical profile was determined with API® 20 C AUX (API® 20 C AUX) (BioMérieux, France).

Genotypic characterization of *Cryptococcus* isolates: For the genotypic characterization of *Cryptococcus* isolates, the methodology described by Jaikel-Viquez and collaborators¹⁰ was followed: extraction of genomic DNA using the heat shock technique. Typing was performed with the RFLP technique of the *URA5* gene, with *Sau96I* and *HhaI* enzymes (Thermo Scientific, USA), using the primers *URA5* (5' ATG TCC TCC CAA GCC CTC GAC TCC 3') and *SJ01* (5' TTA AGA CCT CTG AAC ACC GTA CTC 3'). *Cryptococcus* strains WM 148 (VNI), WM 626 (VNII), WM 628 (VNII), 629 (VNIV), WM 179 (VGI), WM 178 (VGII), WM 161 (VGIII), and WM 779 (VGIV) were used as positive controls from the Westmead Institute for Medical Research, Sydney, Australia.

Results

A total of 66 stool samples were analyzed, from which eight suspicious colonies were isolated. Of these eight, only three were observed to have capsule, urea hydrolysis, and melanin production; therefore, they were preliminarily classified as *Cryptococcus* sp. After biochemical profiling, it was determined that the isolates belonged to the *C. neoformans* and *C. gattii* species complexes. Using growth on CGB, the classification within the *C. neoformans* species complex was corroborated. Using enzymatic restriction, the species and molecular type were identified with the following restriction patterns: VNI (samples 39 and 45) and VNII (sample 58), as shown in Figure 2; being two of the three known molecular types of the *C. neoformans* complex and both belong to the species *C. neoformans sensu stricto*.

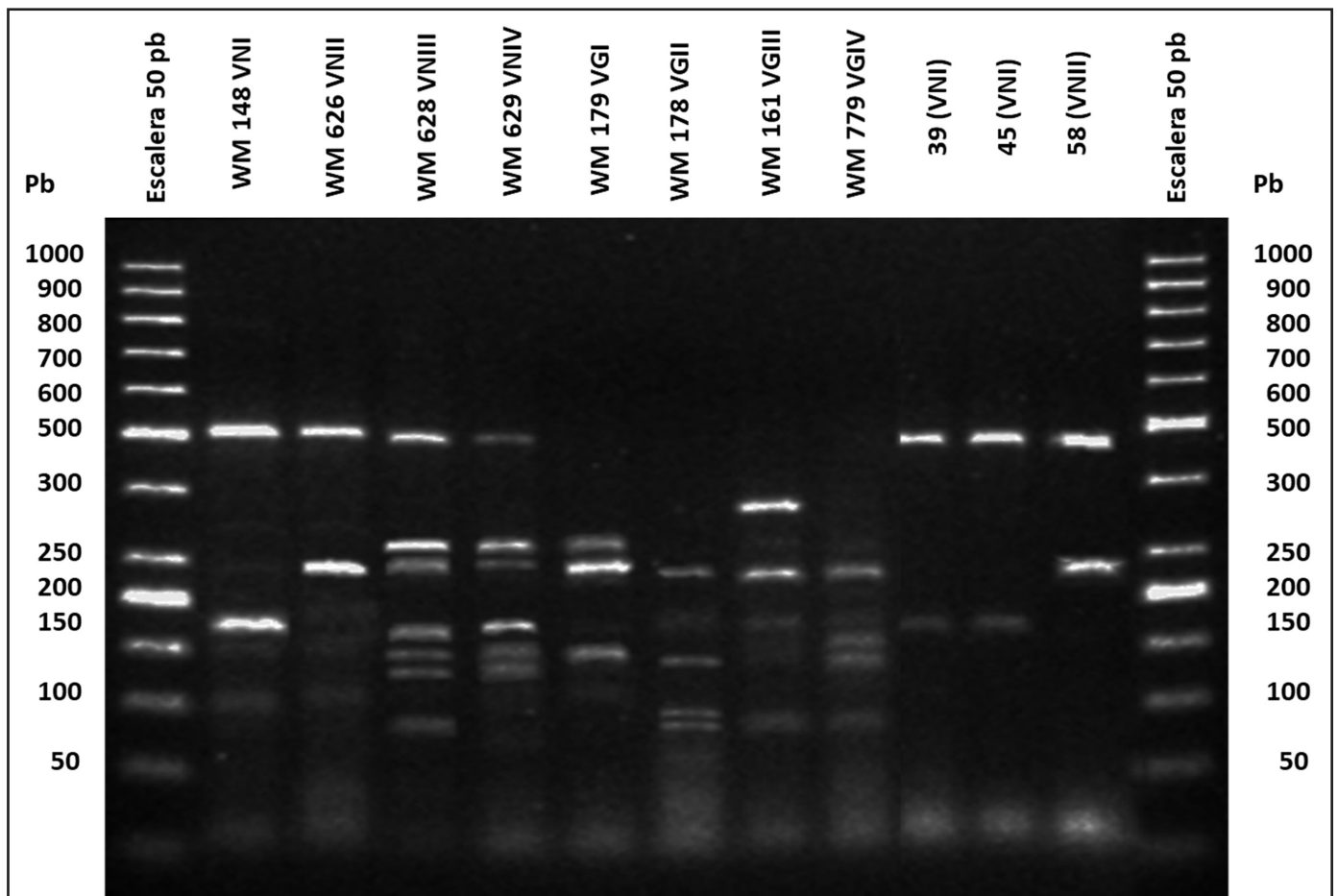


Figure 2. Genotypes of the species complexes *C. neoformans* (VNI-VNIV) and *C. gattii* (VGI-VGIV) and samples 39, 45, and 58 of the excrement of the Feral pigeons (*Columba livia*) collected in public parks and squares in El Salvador, detected according to the enzyme restriction of the *URA5* gene. WM = Westmead Hospital. Molecular weight marker (GeneRuler 50 bp DNA Ladder, Thermo Scientific®).

Of the 11 sites studied, the presence of *C. neoformans sensu stricto* was found in only three: Parque Rafael Campos (department of Sonsonate), Plaza Cívica (La Paz), and Parque Calvario (Santa Ana). One positive sample was found at each site; molecular type NIV was found at Plaza Cívica and El Calvario and NIVII at Rafael Campos Park.

Discussion

The present study identified the presence of *C. neoformans* in 4.5% of the excrement samples of pigeon droppings from public parks. This result is comparable with studies in Peru and Mexico, where it was found that the presence of *C. neoformans* was 13.7% and 5%, respectively.^{12,13}

In a previous study carried out in El Salvador, the fungus was isolated from 36.5% of the samples analyzed.⁸ In this study, the authors analyzed samples from public places and pigeon nests; all samples analyzed from pigeon nests were positive.⁸ However, in the present study, no nest samples were tested.^{8,11,13} It has been noted that studies with a high percentage of *C. neoformans* isolation have obtained samples from nests or caged pigeons, where droppings accumulate and *C. neoformans* are more likely to develop.^{13,14}

As mentioned, this study examined samples from the ground or ledges of monuments and structures in parks and plazas, which are closer to the people who visit these places, for this reason no nest samples were taken. The floor (or exposed surfaces) of parks and squares that receive good solar radiation and high temperatures, like most of the sites sampled for this investigation, are not very conducive to the growth of *C. neoformans* in pigeon droppings.^{15,16,17,18} Environmental factors and the cleanliness carried out in some parks (which prevents the accumulation of droppings) help explain the fact that the presence of this yeast could be confirmed in only three of 66 samples.

As for the genotypes found, the findings of this study are consistent with the fact that the species *C. neoformans* (VNI and VNII) is the most prevalent species of the complex in the environment; moreover, it was the most frequently isolated in a study that analyzed samples from several Latin American countries: 93.3% in Guatemala and 69.6% in Mexico.¹⁹

Its public health relevance is that this strain is the cause of most cases of cryptococcosis in the world and most cryptococcosis in AIDS patients.^{19,20,6}

The findings in this study suggest that factors such as solar radiation and cleanliness do not leave much room for the development of *C. neoformans sensu stricto*. Although in this study there are no environmental data to analyze how these factors influenced the presence of this yeast, immunodeficient people could be at risk when visiting places with the presence of this pigeon and with little solar radiation or cleanliness. Undoubtedly, future studies could evaluate the relationship between this yeast and environmental factors in public places; therefore, it is important to continue in deep studies on public spaces and this pigeon species, to have more objective elements that contribute to improving public health policies in urban centers, where hundreds of individuals of this pigeon species interact daily with the people who live there.

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