Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 6 Issue 9 September 2023

Research Article

Identification of Encapsulated Yeasts of the *Cryptococcus neoformans* Fungus in Public Areas and Surfaces Close to Pigeon (*Columba livia*) Droppings

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DOI: 10.31080/ASMI.2023.06.1292

Received: July 26, 2023

Published: August 11, 2023

Abstract

Cryptococcosis is a systemic fungal infectious disease caused by the *Cryptococcus neoformans/Cryptococcus gattii* complex (serotypes A and D). *Cryptococcus* infections occur by inhalation of blastospores and basidiospores that establish an acute or chronic primary lung infection caused by capsule yeast, especially *C. neoformans*. The urban pigeon (*Columba livia*) is the most important as a reservoir for the fungus *C. neoformans*; this is the cause of fungal meningitis, which causes approximately 278,000 cases and 181,000 deaths each year. In the present study, 100 samples of pigeon excreta were taken, carrying out a cross-sectional, observational and descriptive study. The results showed an identification percentage of 30% positive for *C. neoformans* and 70% corresponding to the presence of other fungal species. Concluding that dovecotes and pigeon feces continue to be one of the main mechanisms of fungal transmission and multiple diseases.

Keywords: Cryptococcosis; *Cryptococcus neoformans*; Pigeons (*Columba livia)*

Introduction

Of the 300 species of fungi recognized as pathogenic for humans, the genus *Candida, Aspergillus, Histoplasma, Pneumocystis* and *Cryptococcus* are the main causes of severe disease in immunocompromised patients [1]. Cryptococcosis is a systemic fungal infectious disease caused by the *Cryptococcus neoformans/ Cryptococcus gattii* complex, serotypes A and D [2]. *Cryptococcus* infections occur by inhalation of blastospores and basidiospores that establish an acute or chronic primary lung infection caused by capsuled yeasts, especially *C. neoformans*; a fungal pathogen that affect to human, which presents central nervous system (CNS) tropism. *Cryptococcus* infections occur by inhalation of blastospores

and basidiospores that establish an acute or chronic primary lung infection caused by capsuled yeasts, especially *C. neoformans*; it causes meningoencephalitis in immunocompromised hosts. *Criptococcus* is found in the environment, mainly in trees and soils contaminated with bird feces, among which is the urban pigeon (*Columba livia*). Since the pigeon plays an important role as a carrier of pathogenic fungi, among the diseases transmitted by pigeons to humans are histoplasmosis, chlamydiosis, salmonellosis, colibacillosis, cryptococcosis, allergic alveolitis, pneumoencephalitis, trypanosomiasis, and tuberculosis [3].

Today it is of great importance to carry out a sanitary control of pigeons, since they are considered potential pests that transmit multiple diseases. In Mexico, no type of sanitary control is carried out, it not required epidemiological surveillance, given the importance in public health is important know the reservoir of this pathogenic microorganism. The possible presence and permanence of the fungus in the digestive tract of pigeons, their distribution and the factors that favor their growth, are topics of great interest for the implementation of sanitary control measures focused on reducing the possibility of transmission of the disease to the population. In Mexico, there is little epidemiological information, diagnosis and susceptibility to different antifungals *in vitro* and Durango is not the exception of the species that cause cryptococcosis. In the municipality of Gómez Palacio, Durango State, there are no previous studies about the presence of *C. neoformans*. In addition, the presence of the pathogen and its impact on the population are unknown.

Therefore, the aim of this research was to identify the presence of encapsulated yeasts of the *Cryptococcus neoformans* fungus in areas and surfaces close to pigeon (*Columba livia*) droppings in public places in Gómez Palacio Durango.

Material and Methods

Transversal, Observational and Descriptive. Public areas of Gómez Palacio Dgo.

Universe and sample

- **Universe:** For the present study, cultures with microbiological isolates were taken into account, in the microbiology laboratory of the Faculty of Chemical Sciences of the city of Gómez Palacio Dgo., in the period of February 2020.
- **Sample:** The number of samples (n) was established for convenience and feasibility of the study. In the present study, 100 microbiological isolates were carried out by the exhaustion streak method on Sabouraud agar with chloramphenicol, in the microbiology laboratory of the Faculty of Chemical Sciences of the city of Gómez Palacio Dgo., in the period of February 2020.

Biosafety protocol

When working with pathogenic and infectious organisms, such as the *Cryptococcus neoformans* fungus, it is necessary to follow biosafety and sanitation protocols within the laboratory, as well as in sample collection.

There are levels, which are ranked from one to four and are selected based on the agents or organisms that are being investigated or worked on in any given laboratory setting.

In the present investigation, we worked with biosafety level 2 (BSL-2) since this level covers laboratories that work with agents associated with human diseases (that is, pathogenic or infectious organisms) that present a risk to health, carrying Perform the following steps as it is:

- Wear appropriate personal protective equipment (PPE), including lab coats and gloves. Eye protection and face shields may also be used as needed.
- Use an autoclave for decontamination and adequate removal of the fungus.
- The laboratory of the Faculty of Chemical Sciences (UJED) in which work was carried out has easily accessible sink facilities and biological hazard warning signs as well as emergency doors.
- Work with a class IIA biosafety cabinet for operator safety.

Biological safety consists of three basic elements to guarantee the adequate containment of biological agents: correct laboratory techniques and practices, the systematic use of equipment and safety means (both are considered primary containment barriers) and the adequate design of laboratory facilities (secondary containment barriers) [4].

Inclusion and exclusion criteria

Inclusion

Areas and surfaces with traces of pigeon (*Columba livia*) excreta.

Exclusion

Areas and surfaces not exposed to pigeon (*Columba livia*) droppings.

Sample collection

For this study, 100 samples of pigeon droppings were taken, carrying out a cross-sectional, observational and descriptive study, the method was carried out in areas and surfaces in 10 x 10 cm quadrants, according to vertices of wind circulation and close to pigeon droppings (*Columba livia*). The 100 samples were collected, 20 from each public area, Guadalupe Church, Main Square Gomez Palacio, Faculty of Engineering, Sciences and Architecture (FICA), UJED Library and Morelos Park. The excrement was obtained with swabs impregnated with saline solution, using Stuart medium as a transport medium. The tubes with the samples were labeled, transported and stored in the microbiology laboratory.

Sample processing

Samples were isolated and processed by the striae depletion isolation technique with 0.9% isotonic saline solution on Sabouraud fungal and yeast agar with 0.25 ml of chloramphenicol to inhibit bacterial growth and thus avoid competition with fungal growth, and yeast agar with 0.25 ml of chloramphenicol to inhibit bacterial growth and thus avoid competition with fungal growth.

Incubation was carried out at 30ºC, observing daily until detecting yeast-like growth. Samples that presented growth characteristics consistent with mucoid, white and creamy colonies were considered positive for *Cryprococcus* growth. From the growth with these characteristics, a smear was made where a drop of India ink was placed for microscopic observation at 40x magnification to identify if it was *Cryptococcus neoformans* as observed in (Figure 1) the process of smearing with India ink.

Samples positive for *Cryptococcus neoformans* were replanted and identified using the lactophenol blue technique and the nitrate reduction test.

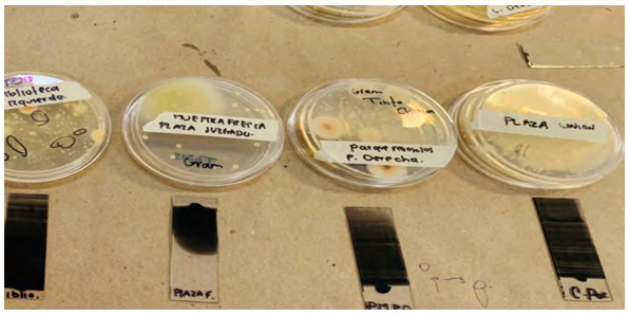

Figure 1: India ink staining on 40x saboraud agar growth.

Techniques and instruments

Stuart microbiological transport medium

A transport medium for microbiology is a culture medium capable of keeping microorganism strains alive for a long period of time, which keeps the microorganisms alive without altering their concentration. Stuart's medium allows the preservation and transport of an infinite number of pathogenic microorganisms such as *Cryptococcus*, *Shigella* spp, *Salmonella* spp, *Streptococcus* spp, *Neisseria gonorrhoeae*, among others. One of the characteristics of this medium is that it hinders enzymatic autolyzing reactions, in addition to the absence of a nitrogen source prevents the accompanying flora from proliferating.

Formula for one liter of purified water:

- Sodium Tioglycolate 1.0 g
- Sodium Glycerophosphate 10.0 g
- Calcium chloride 0.1 g
- Methylene blue 0.002 g
- Bacteriological Agar 3.0 g

Procedure

- Suspend 14.1 g of the medium in 1 liter of purified water.
- Heat with gentle agitation until complete dilution of the powder, taking care that the medium does not boil.
- Dispense into glass tubes, cap, and autoclave at 121°C for 15 min.

Sabouraud Dextrose agar medium with Chloramphenicol

Formula for one liter of purified water:

- Peptone 5.0 g
- Triptein 5.0 g
- Glucose 40.0 g
- Chloramphenicol 0.05 mL
- Agar 15.0 g

Procedure

- Suspend 65 g of the powder in 1 liter of purified water.
- Rest for 5 min and mix until uniform.
- Heat, stirring frequently, without boiling, for 1 min until completely dissolved.
- Autoclave at 115-121ºC for 15 min.
- Distribute in sterile Petri dishes.

Note: keep in a cool place, as exposure to heat increases the hydrolysis of the components.

Identification techniques

India ink smear to observe capsules

It is a quick and useful diagnostic technique, with Chinese ink staining being common, in which yeasts are observed surrounded by a colored capsule that forms a clear halo (5).

Procedure

On a slide place:

- Chinese ink 1 drop.
- Distilled water or physiological solution 1-2 drops.
- Emulsify the sample.
- Place coverslips.

Staining with lactophenol blue

Procedure

- Take a pure culture of the fungus whose structures you want to observe.
- Place a drop of lactophenol blue on a slide.
- It is carefully taped to the end of the cold, sterilized platinum loop.
- The platinum loop is brought closer to the most superficial part of the fungal colony and very carefully touches the culture, exactly where the adhesive tape sticks.
- Then it is taken to the slide and placed just over the drop of lactophenol blue, taking care that the tape, it is perfectly extended and lubricated with the dye. Remove the platinum loop.
- Another drop of lactophenol blue is placed on the tape and a coverslip on top. Too much pressure should not be exerted so as not to destroy the structures of the fungus. The dye should be left to act for 3 to 4 min.
- After this time, the preparation is ready to be observed under a microscope at 10x or 40x magnification.

The structures of the fungus, in general, are stained blue, with the exception of dematiaceous fungi that will retain their characteristic brown coloration of this type of fungus.

Gram stain

The technique consists of the following steps:

- Make a smear and fix it with heat.
- Cover with crystal violet for one min, and then wash lightly with distilled water.
- Cover with Lugol's for one min and wash with distilled water.
- Decolorize with 10 to 20 drops of a 50% alcohol-acetone solution, and wash lightly with distilled water.
- Cover with safranin for 30 s, wash, allow drying, and observing at 100 x.

Identification by biochemical and enzymatic criteria

Nitrate reduction test

Procedure

- With the help of a swab, take two to three isolated colonies from a culture of 48-72 h of growth in any usual culture medium.
- The inoculated swab is pressed firmly against the bottom of an empty tube to dislodge microorganisms contained in the cotton fiber.
- Incubate the tube with the swab at 45ºC for ten min.

- Remove the swab and add to the tube two drops of alpha-naphthlamide (reagent A) and two drops of sulfanilic acid (reagent B).
- Reintroduce the swab into the tube to absorb the reagents.

Interpretation

Development of a bright red color on the swab is positive, negative only when the swab retains the color of the colony.

- **Positive:** *Cryptococcus albidus* var albidus.
- **Negative:** *C. neoformans.*

Results

In the present study, 100 samples of pigeon (*Columba livia*) feces were analyzed, which were collected from various areas of the city of Gómez Palacio Durango, Mexico. According to the results obtained from the total samples collected, 30% were positive for C. neoformans (Figure 2). The other 70% corresponds to the presence of other species of fungi, among which are: *Penicillium* spp, *Rhizopus* spp, and *Aspergillus flavus* (Figure 3). Figure 4 shows the percentage of fungi identified.

Figure 2: Colonies of *C. neoformans* on Sabouraud agar with chloramphenicol.

Figure 3: Sabouraud agar culture with growth of different types of fungi.

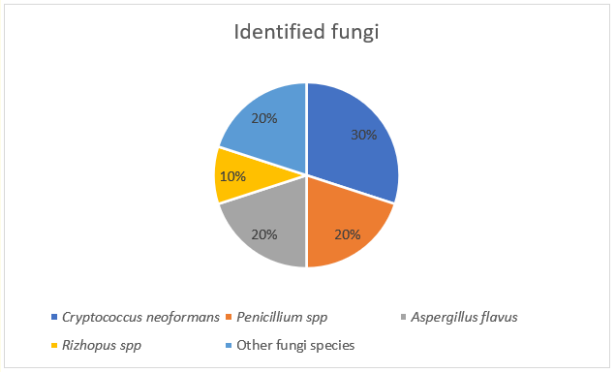

Figure 4: Percentage of fungi identified.

In most areas, exposure to light, air circulation, and the permanence of excreta in the environment were high. Humidity in most of the sampling areas was low (60%). 80% of the samples taken were not fresh and the vast majority were taken dry, outdoors, mainly from the ground. Table 1 shows the identification of *C. neoformans* in the municipality of Gómez Palacio. The identification percentage of the 30 positive samples obtained is shown in the Figure 5.

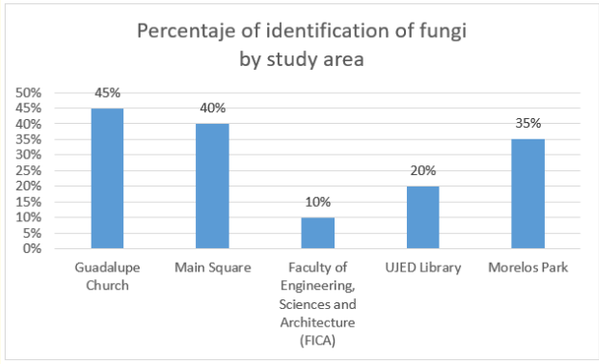

Figure 5: Identification percentage of fungi by study area.

Positive samples and percentage of identification			
Site	**Number**	**Positive**	**Percentage**
Guadalupe Church	20	9	45%
Main Square Gómez Palacio	20	8	40%
Faculty of Engineering, Sciences and Architecture (FICA)	20	2	10%
UJED Library	20	4	20%
Morelos Park	20	7	35%
Total	100	30	30%

Table 1: Identification of *Criptococcus neoformans* in the municipality of Gómez Palacio.

During the study, other types of fungi of clinical importance were identified that may be of interest in other investigations, as shown in the table 2.

Place	Location	Number of samples	Number of positive samples	Identified fungus
Guadalupe Church	Right side (floor)	5	3	*C. neoformans Aspergillus* spp *Rhizopus* spp
	Left side (floor)	5	2	*C. neoformans Aspergillus* spp
	In front (floor)	5	2	*C. neoformans Candida, Aspergillus*
	Air apparatus	5	2	*C. neoformans Candida, Penicillum*
Main Square Gómez Palacio	Kiosk	5	2	*C. neoformans Aspergillus* spp
	North Quadrant (floor)	5	1	*C. neoformans,* otro tipo de hongos
	South quadrant (floor)	5	1	*C. neoformans Rhizopus* spp
	Concrete walls	5	4	*C. neoformans Candida, Penicillum*
Faculty of Engineering, Sciences and Architecture (FICA)	Benches	5	1	*C. neoformans Aspergillus* spp
	Air apparatus	5	0	*Candida, Penicillum,*
	Playing fields	5	1	*C. neoformans Candida, Penicillum*
	Parking lot	5	0	*Aspergillus*
UJED Library	Air apparatus	5	1	*C. neoformans Candida, Rhizopus* spp
	Left side bench	5	2	*C. neoformans,* otro tipo de Hongos
	Windows Right Side	5	1	*C. neoformans Aspergillus*
	Rear side	5	0	*Aspergillus, Candida, Rhizopus*
Morelos Park	Benches	5	2	*C. neoformans,* otro tipo de hongos
	Concrete walls	5	3	*C. neoformans Candida, Penicillum*
	South quadrant (floor)	5	1	*C. neoformans, Candida*
	North Quadrant (floor)	5	1	*C. neoformans, Penicillum,* otro tipo de hongos

Table 2: Types of Fungi identified in the different sampled areas.

Discussion

The study of the *Cryptococcus neoformans* fungus is of great importance because in recent decades, the domestic pigeon has increased in population together with urban growth and this represents a problem for public health since its feces can maintain viable pathogenic microorganisms, such as *Cryptococcus*. This study evidenced the isolation of *C. neoformans* in dry pigeon droppings, confirming that this substrate is an important source of the fungus in the urban environment. These results agree with an investigation carried out in the present year 2022 in the Democratic Republic of the Congo in the which determined that the *Cryptococcus neoformans*/*C. gattii* are the main etiological agents of cryptococcosis, based on epidemiology, geographic distribution, ecological niches, clinical presentation, therapeutic and genetic differences.

In this sense, the accumulation of pigeon feces in public areas represents a risk of contagion since it acts as a transmission vehicle. According to the results obtained, it was possible to identify the fungus *Cryptococcus neoformans*, although in various areas its identification was scarce, it was determined, that in the Church of Guadalupe and in Main Square the presence of the fungus was higher compared to the other sites UJED Library, Faculty of Engineering, Sciences and Architecture (FICA), and Morelos Park.

Vallejo., *et al*. (2016) document that bird droppings are perfect reservoirs for *Cryptococcus*, because the temperature of the birds prevents the growth of the specie. Therefore, when they are expelled in the droppings and when the temperature drops, they are able of proliferate, and the spores are disseminated by the wind once the excreta is dry, in addition to providing nutrients for their survival [3].

In another study, Iyappanand Karthikeyan (2011) described that the composition of bird excreta changes according to the diet since the positive extracts they obtained in their research came from pigeons fed only with corn, while the pigeons from which they were obtained the negative extracts were fed a variety of grains [6].

On the other hand, according to the results obtained by Nielsen., *et al.* (2007), it is determined that *C. neoformans* grows better in pigeon guano than in a yeast-based culture medium, which agrees with the results obtained in the present investigation and which corroborates that the nutritional composition can provide a favorable environment for the growth of these fungi [7].

It is worth mentioning that in the present investigation it was not possible to isolate *C. neoformans* in fresh pigeon droppings, which coincides with the findings of an investigation already carried out by Nunes Calumby in 2021. In that research they mention that it was not possible to isolate *C. neoformans* in droppings fresh, and that can be justified by the intense bacterial proliferation in these excrements, promoting a change in the pH, making it alkaline, inhibiting the growth of yeast [8].

According to Ribeiro (2019), dry excrement offers a more favorable organic substrate for the development of the fungus, rich in nitrogen, with a lower concentration of bacteria, which reduces competition for growth [9].

Regarding the isolated strains, it was found that 70% corresponds to the presence of other species of fungi, among which are: *Penicillium* spp, *Rhizopus* spp, and *Aspergillus flavus*. This indicates that pigeon feces are a large reservoir of different pathogenic fungi and bacteria, as mentioned in a study carried out in 2021 in the city of Quito, Ecuador. Where they statistically determined the possible role of pigeon feces as fomites, for the determination of pathogenic bacteria such as *Salmonella* spp, in this study the *Salmonella* data reported indicate the existence of the microorganism in the fecal matter of pigeons (*Columbia livia*), which represents the first step to determine a public health problem [10]. What is alarming for the human being because these are animals that are found in squares and parks where the traffic of people is high and where many of them are dedicated to the sale of street food? This implies the presence of animals whose biological waste such as feces could spread and contaminate the soil, and food consumed by the population.

The strength of the present study was the analysis of the identification of the fungus in public areas close to pigeon (*Columba livia*) droppings. A 6-month follow-up was performed compared to the 1-year follow-up period in most studies because the study is cross-sectional, observational, and descriptive. The results were obtained from the different sampled areas, in which the presence predominated in some places more than in others. In summary, we analyzed 100 samples collected from the different areas already

mentioned, confirmed by Sabouraud agar culture with antibiotics in a period of 6 months in the city of Gómez Palacio Durango, obtaining as a result 30% of positive samples for *Cryptococcus neoformans* and 70% for other species of fungi.

Conclusion

According to the results obtained, it is concluded that the dovecotes and the feces of the pigeons continue to be one of the main mechanisms of transmission of fungi such as *Cryptococus neoformans*, *Penicillium* spp, *Aspergillus flavus* and *Rizhopus* spp, in healthy people, as well as in immunosuppressed patients. That is why today it is of great importance to carry out sanitary control measures to reduce the possibility of transmission of diseases from birds to the population, since pigeons are considered potential pests that transmit multiple diseases, which is alarming because. They are animals that travel long distances due to their ability to fly, so the microorganism can spread more easily. Although with minimal results, we obtained positive samples in the soil near the droppings, these findings show the need for measures to control these birds in places of public circulation, such as squares and parks, as well as the application of sanitation and cleaning practices of droppings.

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