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Isolation of saprophytic *Cryptococcus neoformans* from Puerto Rico: Distribution and variety

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Abstract

Until the present decade, no studies had been conducted in Puerto Rico on the saprophytic distribution and variety of *Cryptococcus neoformans*. Samples (522) of pigeon droppings from 14 western towns were tested for the presence of *C. neoformans*. The yeast was recovered from 24.7% (129 isolates) of the samples, representing 10 of the 14 towns studied. All environmental isolates were identified as *C. neoformans* var. *neoformans* using canavanine-glycine-bromthymol blue (CGB) agar. The yeast was isolated from 79.4% of the samples in one town, Isabela. The average number of yeast cells isolated from sites within this municipality was 5.1×10^5 per gram of pigeon droppings. This was 2.6 times the average number of yeast cells of *C. neoformans* isolated from sites in other towns. In addition, the yeast was isolated from four patients with the acquired immune deficiency syndrome (AIDS), each of whom died of cryptococcal meningitis. Each of these poorly encapsulated isolates was identified as *C. neoformans* var. *neoformans* using CGB agar. The results of this investigation demonstrate that *C. neoformans* var. *neoformans* is prevalent in Puerto Rico.

Introduction

Cryptococcus neoformans is an encapsulated yeast with worldwide distribution. This opportunistic fungal pathogen is primarily associated with soils containing pigeon and other bird droppings [1,4,19,21,22]. The yeast is recognized as the etiologic agent of cryptococcosis, a subacute to chronic infection that most often affects the lungs and central nervous system [15].

The fungus grows on artificial culture media and *in vivo* as polysaccharide encapsulated yeast cells ranging in size from 4–20 μm. Four major serotypes of *C. neoformans* have been described based on antigenic differences of the capsular material; a possible fifth serotype, i.e., serotype A–D, also has been proposed [7]. Kwon-Chung *et al.* [10] established morphologic, biochemical and genetic similarities between serotypes A and D, and between serotypes B and C. They recognized two varieties of *C. neoformans*: var. *neoformans* corresponding to serotypes A and D, and var. *gattii* corresponding to serotypes B and C. Further information related to the biochemical

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serogrouping of *C. neoformans* recently has been published by Shadomy *et al.* [18].

Serotype A and, with some exception, serotype D occur worldwide and have been cultured from clinical specimens and from environmental sources, including pigeon droppings. Serotypes B and C have a more restricted distribution; these isolates are obtained only from clinical specimens most frequently from Southern California and from tropical or subtropical areas such as Southeast Asia, although they are not limited exclusively to these biomes [1, 9, 10]. Ellis [3] recently reported an unusually high incidence of *C. neoformans* var. *gattii* in patients from the Northern Territory (95%) and from South Australia (65%).

The purpose of this investigation was to study the distribution and variety of *C. neoformans* in Puerto Rico, a tropical island in the Caribbean basin.

Materials and methods

Random samples (522) of dry pigeon droppings were collected from different locations within 14 municipalities in Western Puerto Rico (Fig. 1). The samples were cultured for the number of viable cells of *C. neoformans* per gram as described previously [16, 17]. Briefly, duplicate one gram samples from each location were placed in bottles containing 99 ml of sterile saline supplemented with gentamicin and chloramphenicol to final concentrations of 8 mg/ml and 20 mg/ml, respectively. The mixture was allowed to stand for 10 minutes and then shaken vigorously for five minutes. Samples were serially diluted tenfold and 0.1 ml of each dilution was spread onto the surface of niger seed (*Guizotia abyssinica*) agar supplemented with the previously mentioned antibiotics. The plates were incubated at 25 °C and colonies of *C. neoformans* enumerated after 72–96 hr. The results were expressed as the average number of viable yeast cells per gram of droppings. The species identification was done according to published criteria [11].

The four clinical isolates of *C. neoformans* were obtained from the cerebrospinal fluid of patients diagnosed as having the acquired immune deficiency syndrome (AIDS). Each patient lived in the city of Mayaguez. The isolates were maintained on Sabouraud dextrose agar slants until tested for variety as described below.

The varietal status of each isolate of

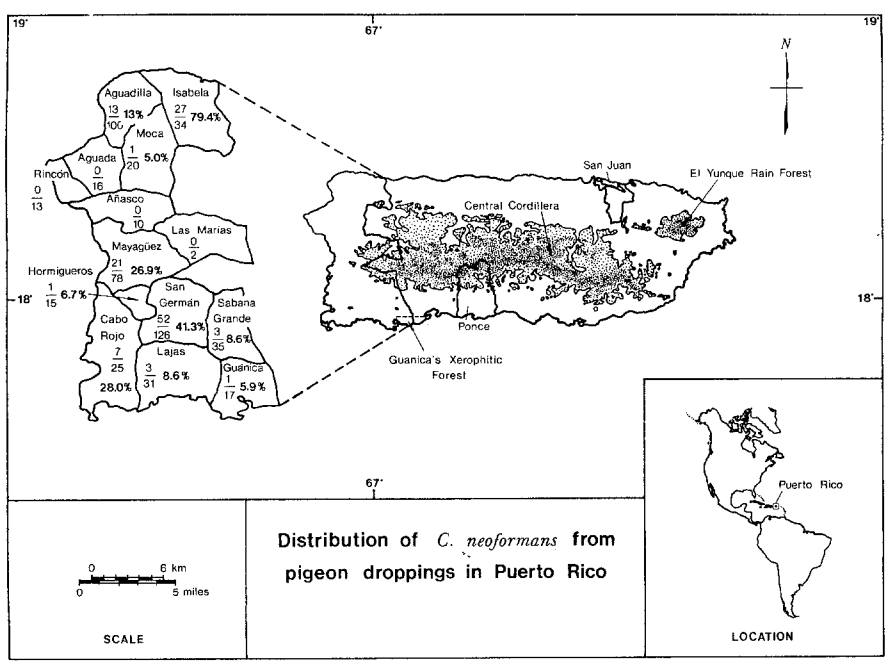

Fig. 1.

C. neoformans was determined using the canavanine-glycine-bromthymol blue agar described by Kwon-Chung *et al.* [10]. *Cryptococcus neoformans* isolates of known serotype deposited with the American Type Culture Collection (ATCC: 28958, 34871, 32608, 32609, 34868, 3502) were used as controls for this system.

Results and discussion

Pigeon droppings (522 samples) from 14 municipalities in Western Puerto Rico were assayed for the presence and quantity of *C. neoformans*. The yeast was recovered from 24.7% (129 isolates) of the samples, representing 10 of the 14 municipalities studied (Fig. 1, Table 1). An average of 1.94×10^5 yeast cells/g of droppings was isolated from the sites studied. The fungus was isolated from 79.4% of the samples from the municipality of Isabela, with an average number of 5.1×10^5 cells/g of droppings, 2.6 times the average number of *C. neoformans* cells obtained from sites in other towns. The greatest number of cells/g of droppings (2.1×10^6) was obtained from a sample collected at a beach area known as El Seco in Mayaguez. All *C. neoformans* isolates were variety *neoformans* as determined by CGB agar.

Table 1. Distribution of *C. neoformans* from pigeon droppings in Puerto Rico.

	Town	Number of samples (% of total)		Number of *C. neoformans* isolates (% recovery)	
1.	Isabela	34	(6.5)	27	(79.4)
2.	San Germán	126	(24.1)	52	(41.3)
3.	Cabo Rojo	25	(4.8)	7	(28.0)
4.	Mayaguez	78	(14.9)	21	(26.9)
5.	Aguadilla	100	(19.2)	13	(13.0)
6.	Lajas	31	(5.9)	3	(8.6)
7.	Sábana Grande	35	(6.7)	3	(8.6)
8.	Hormigueros	15	(2.9)	1	(6.7)
9.	Guánica	17	(3.3)	1	(5.9)
10.	Moca	20	(3.8)	1	(5.0)
11.	Las Marías	2	(0.4)	0	
12.	Rincón	13	(2.5)	0	
13.	Aguada	16	(3.1)	0	
14.	Añasco	10	(1.9)	0	
	Total	522		129	24.7

Our findings demonstrate that *C. neoformans* is prevalent in Western Puerto Rico and that its geographical occurrence does not differ noticeably from what has been reported in other countries. However, it should be noted that the average frequency of occurrence (24.7%), as well as the maximum frequency of occurrence in the town of Isabela (79.4%), appear to be the highest published to date.

Our results suggest that many inhabitants of this island are frequently exposed to *C. neoformans*; however, surprisingly, only four cases of cryptococcosis have been diagnosed since 1981 at the Mayaguez Medical Center, a primary public health care facility that serves the western portion of the island. Each of these four cases were patients diagnosed as having AIDS, and each died of cryptococcal meningitis, caused by isolates of poorly encapsulated *C. neoformans* var. *neoformans*. This apparently low frequency of cryptococcosis among Puerto Ricans is the subject of another study in which we are investigating the concept of an endogenous source or a carrier state for cryptococcosis as has been suggested previously [6, 13].

The geographic distribution of this fungus has been of great interest in recent years, particularly since the recognition that *C. neoformans* var. *gattii* is absent in soils. A study by Kwon-Chung *et al.* [9] showed that the variety *gattii* was isolated exclusively from clinical specimens, and largely from patients in Southern California and Southeast Asia. In the same study, these authors reported that among isolates of *C. neoformans* in North America, excluding Southern California, 6.7% were variety *gattii*. Other reports [3, 5, 12] support the concept of a potentially broader distribution of *C. neoformans* var. *gattii* than that currently observed.

Isolates of *C. neoformans* var. *gattii* were not found in our environmental or clinical samples. These data support earlier studies by other investigators that suggest a restricted distribution of

this variety and the almost exclusive absence of variety *gattii* as an infectious agent in AIDS patients. Among recent reports in the literature [2, 8, 14, 20], variety *gattii* was isolated from only one of more than 100 AIDS patients [8]. However, it should be emphasized that the present investigation was limited to Western Puerto Rico. A more comprehensive study involving other geographic areas on this tropical island that have markedly varied climatologic and ecologic characteristics is now in progress.

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