



Original article

Molecular characterization and antifungal susceptibility of *Cryptococcus neoformans* strains collected from a single institution in Lima, Peru



Vilma Bejar^a, Mercedes Tello^{b,*}, Ruth García^b, José M. Guevara^a, Sofia Gonzales^a, German Vergaray^a, Esther Valencia^a, Enma Abanto^a, Alex G. Ortega-Loayza^c, Ferry Hagen^{d,e}, Ericson L. Gutierrez^{f,g}

^a Instituto de Medicina Tropical "Daniel Alcides Carrión", Facultad de Medicina, Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru

^b Facultad de Ciencias Biológicas, UNMSM, Lima, Peru

^c Department of Dermatology, Virginia Commonwealth University, Richmond, VA, USA

^d CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands

^e Department of Medical Microbiology and Infectious Diseases, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands

^f Unidad de Análisis y Generación de Evidencia en Salud Pública, Instituto Nacional de Salud, Lima, Peru

^g Facultad de Medicina, Universidad de San Martín de Porres, Lima, Peru

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ABSTRACT

Background: Cryptococcosis is a fungal infection with a worldwide distribution, mainly caused by *Cryptococcus neoformans* and *Cryptococcus gattii*.

Aims: To molecularly characterize the mating-types, serotypes, genotypes and antifungal susceptibility profiles of a set of retrospectively isolated *C. neoformans* strains from Lima, Peru.

Methods: A set of 32 *Cryptococcus* spp. strains from the Institute of Tropical Medicine of the National University of San Marcos, Lima, Peru, were included in this retrospective study. Twenty-four strains were isolated from patients, while the remaining 8 were isolated from the environment.

Results: Using conventional PCR, 27 (84.4%) of the isolates were identified as *C. neoformans* var. *grubii* mating-type alpha and serotype A. Using the AFLP fingerprinting, it was shown that 16 (50%) of the *C. neoformans* strains were genotype AFLP1, 13 (40.6%) were genotype AFLP1B, 2 (6.3%) were genotype AFLP2, and 1 (3.1%) was found to be a hybrid between both *C. neoformans* varieties (genotype AFLP3). The antifungal susceptibility profiles for amphotericin B, fluconazole and voriconazole showed that all the 32 *C. neoformans* are sensitive to these antifungal compounds.

Conclusions: In this study we observed that *C. neoformans* var. *grubii* (AFLP1 and AFLP1B) and *C. neoformans* var. *neoformans* (AFLP2) were the only cryptococcal varieties involved. All strains were found to be sensitive to the antifungals tested, results that are consistent with those found in the international literature.

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Caracterización molecular y sensibilidad antifúngica de cepas de *Cryptococcus neoformans* provenientes de una institución de Lima, Perú

RESUMEN

Antecedentes: La criptococosis es una infección fúngica de distribución mundial, causada principalmente por *Cryptococcus neoformans* y *Cryptococcus gattii*.

Objetivos: Determinar el tipo de apareamiento, los serotipos, los genotipos y la sensibilidad antifúngica de las cepas de *C. neoformans* provenientes de Lima Perú.

Métodos: Se analizaron 32 cepas de *Cryptococcus* spp. del Instituto de Medicina Tropical de la Universidad Nacional Mayor de San Marcos de Lima, Perú. Veinticuatro cepas provenían de pacientes y 8 cepas fueron obtenidas de muestras ambientales.

Palabras clave:

Cryptococcus neoformans

Genes del tipo sexual de los hongos

Serotipificación

Genotipo

* Corresponding author.

E-mail address: mechetr@hotmail.com (M. Tello).

Resultados: Mediante la técnica de reacción en cadena de la polimerasa se determinó que 27 (84,4%) aislamientos eran *C. neoformans* var. *grubii*, serotipo A, con el tipo de apareamiento alfa. Asimismo, empleando la técnica AFLP, se determinó que 16 (50%) cepas de *C. neoformans* eran del genotipo AFLP1 y 13 (40,6%) del genotipo AFLP1B; además, 2 (6,3%) eran del genotipo AFLP2 (*C. neoformans* var. *neoformans*) y 1 (3,1%) resultó ser un híbrido entre ambas variedades (AFLP3). Los perfiles de sensibilidad antifúngica para anfotericina B, fluconazol y voriconazol mostraron que las 32 cepas de *C. neoformans* eran sensibles al panel de antifúngicos.

Conclusiones: En el presente estudio observamos que *C. neoformans* var. *grubii* (AFLP1 y AFLP1B) y *C. neoformans* var. *neoformans* (AFLP2) fueron las variedades encontradas. Además, todas las cepas resultaron sensibles al panel de antifúngicos, en concordancia con la literatura internacional.

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The genus *Cryptococcus* includes more than 100 species, of which only the *Cryptococcus neoformans*/*Cryptococcus gattii* complex is considered a real human pathogen.²³ *C. neoformans* is divided into the two varieties *C. neoformans* var. *grubii* (serotype A; genotypes AFLP1, AFLP1A and AFLP1B), *C. neoformans* var. *neoformans* (serotype D; genotype AFLP2), and hybrids between both varieties (serotype AD; genotype AFLP3).³ *C. gattii* was until a decade ago considered a variety of *C. neoformans*, but was finally raised to the species level based on physiological, ecological, epidemiological and genetic differences.²⁴ *C. gattii* can be divided into five genotypes: AFLP4 (serotype B), AFLP5 (serotype C), AFLP6 (serotype B), AFLP7 (serotype C) and AFLP10 (serotype B).¹⁹ Hybrids between *C. gattii* and *C. neoformans* have recently been described.¹

C. neoformans var. *grubii* is globally distributed, and can mainly be found in soil contaminated with bird droppings.¹² *C. neoformans* var. *neoformans* is commonly encountered in Europe but less frequently observed to cause disease outside this continent. Both varieties primarily affect immunocompromised patients.³⁰ *C. gattii* is mainly found in tropical and subtropical climates, and has been isolated from a variety of natural sources, mostly affecting immunocompetent patients.¹⁶ The incidence of cryptococcosis in HIV-infected patients has decreased dramatically in developed countries after the HAART era; unfortunately, in developing countries without access to HAART the incidence is considerable.³⁰ Moreover, in Peru, the majority of the patients with HIV are homosexual men; however, the incidence is increasing in heterosexuals. In 2000–2010, the prevalence of treated HIV-positive patients with cryptococcosis was 14% (259/1848) at the Dos de Mayo National Hospital (one of the largest in the country), being neurocryptococcosis the most frequent diagnosis.²⁰ The incidence of neurocryptococcosis in HIV-infected patients in Peru before the HAART era was 5.6%¹⁰ and, as was observed globally, it decreased after the implementation of HAART. However, neurocryptococcosis still represents a public health problem. Despite the administration of HAART, some patients start the treatment in advanced stages of the disease, as in many cases the diagnosis of HIV-infection is performed when the patient presents an opportunistic disease such as cryptococcosis.

The objective of this study was to determine the mating-types, serotypes, genotypes and antifungal susceptibility profiles of *C. neoformans* strains available in the culture collection of the Institute of Tropical Medicine from the National University of San Marcos, Lima, Peru.

Materials and methods

A set of 32 *Cryptococcus* spp. strains from the Institute of Tropical Medicine from the National University of San Marcos (ITM-UNMSM), Lima, Peru, was included in this retrospective study. The

ITM-UNMSM is one of the main centers for tropical infectious diseases research in Peru. The samples were collected between January 2008 and December 2012. Clinical samples came from the Dos de Mayo National Hospital in Lima, and the Daniel Alcides Carrión National Hospital in Callao. Twenty-four strains came from patients, while the remaining eight were years ago isolated from the local environment. Of the 24 clinical strains, 23 came from HIV-infected patients and one from an immunocompetent individual. In all the clinical cases, the samples were taken from the cerebrospinal fluid. The environmental strains came from pigeon droppings collected from around the university campus of the National University of San Marcos.

Mating-type, serotype and genotype determination

The mating-type and serotype were molecularly determined using four conventional PCRs that specifically amplify the *STE20a* and *STE20α* allele of serotype A and D strains.² The genotype was determined by using amplified fragment length polymorphism (AFLP) fingerprinting as previously described.¹⁷ The reference strains CBS8710 (AFLP1; αA), CBS9172 (AFLP1; αA), CBS11261 (AFLP1A; αA), CBS10083 (AFLP1B; αA), CBS10511 (AFLP2; αD), CBS10513 (AFLP2; αD), CBS10080 (AFLP3; αAαD), CBS10078 (AFLP4; αB), CBS10081 (AFLP5; αB), CBS10082 (AFLP6; αB), CBS10101 (AFLP7; αC) and IHEM14941 (AFLP10; αB) were included to determine the genotype. A dendrogram of the Peruvian strains was constructed using single linkage clustering in combination with the Pearson correlation using BioNumerics version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).

Antifungal susceptibility

The antifungal susceptibility was evaluated by antifungal disk diffusion testing according to the Clinical and Laboratory Standards Institute.⁸ According to these guidelines, Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml of methylene blue (MHM) was used. The antifungal panel consisted of (all from HiMedia Laboratories, Mumbai, India) fluconazole (25 µg), voriconazole (1 µg) and amphotericin B (20 µg). The plates were incubated at 35 °C. Quality controls were tested in the same way with *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6528.

The inhibition zones were measured in millimeters at 48 and 72 h. Breakpoints for fluconazole and voriconazole were determined according to Khan et al.²² The breakpoints for fluconazole were susceptible (S) ≥ 19 mm, susceptible dose-dependent (SDD) 15–18 mm, and resistant (R) ≤ 14 mm. For voriconazole breakpoints were (S) ≥ 17 mm, SDD 14–16 mm, and R ≤ 13 mm. The set breakpoints for amphotericin B, according to Da Silva et al.,¹¹ were S > 10 mm, R < 10 mm.

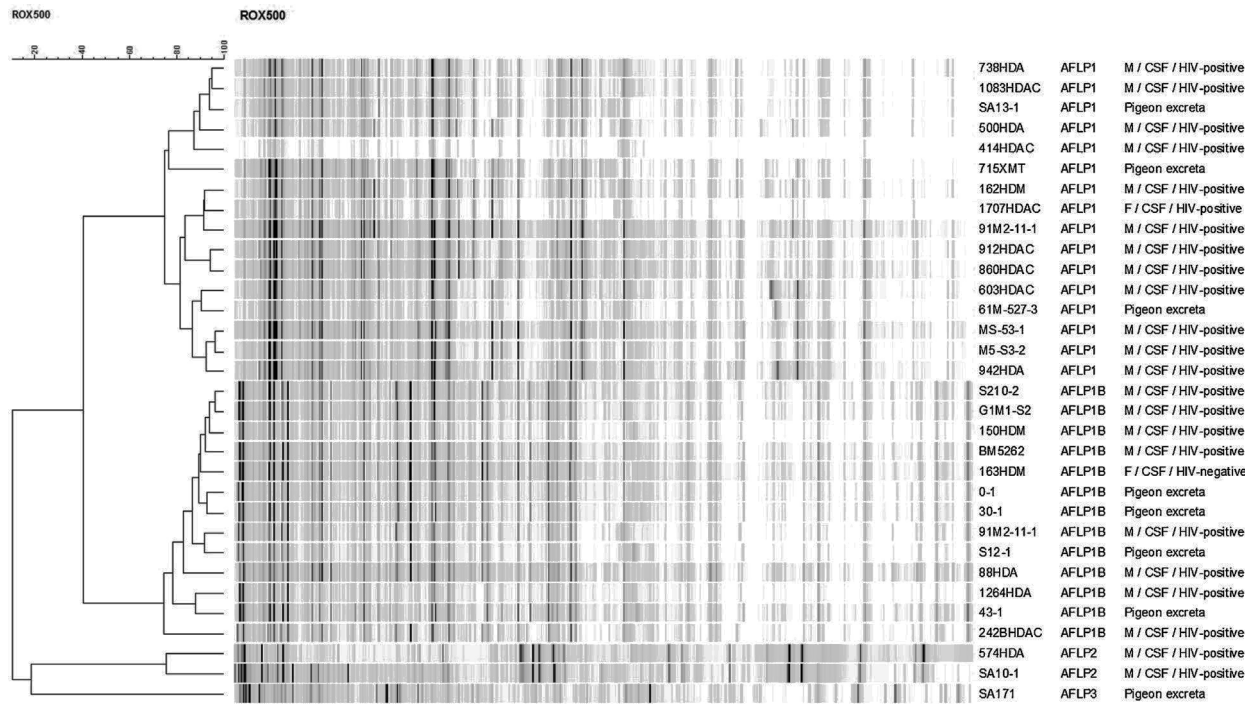


Fig. 1. Results of AFLP genotyping, mating-typing and serotyping of 32 *Cryptococcus neoformans* strains.

Table 1
Distribution of *Cryptococcus neoformans* mating-types, serotypes and genotypes.

Isolates	Serotype		Mating-type		AFLP genotype			
	Serotype A	Non-typeable	α	Non-typeable	AFLP1	AFLP1B	AFLP2	AFLP3
Clinical	22 (91.7%)	2 (8.3%)	22 (91.7%)	2 (8.3%)	13 (54.2%)	9 (37.5%)	2 (8.3%)	–
Environmental	5 (62.5%)	3 (37.5%)	5 (62.5%)	3 (37.5%)	3 (37.5%)	4 (50%)	–	1 (12.5%)
Total	27 (84.4%)	5 (15.6%)	27 (84.4%)	5 (15.6%)	16 (50%)	13 (40.6%)	2 (6.3%)	1 (3.1%)

Results

Mating-types, serotypes and genotypes

Twenty-seven (84.4%) strains out of the 32 strains tested were found to be alpha mating-type and serotype A by using the conventional PCR approach. This was confirmed by AFLP fingerprinting since these 27 strains were found to be *C. neoformans* var. *grubii* genotype AFLP1 (*n* = 16; 50%) and AFLP1B (*n* = 11; 34.4%). Five strains (15.6%) were untypeable by PCR but were found by AFLP fingerprinting to belong either to genotype AFLP1B (*n* = 2; 6.2%), AFLP2 (*n* = 2; 6.2%) or AFLP3 (*n* = 1; 3.1%), representing *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and the hybrid between both varieties, respectively (Fig. 1). Of the clinical *C. neoformans* var. *grubii* strains, 13 (54.2%) were genotype AFLP1 and 9 (37.5%) were AFLP1B; 2 (8.3%) were *C. neoformans* var. *neoformans*. Three (37.5%) strains out of the 8 (25%) environmental samples fell within the genotype AFLP1-cluster, 4 (50%) in the genotype AFLP1B-cluster and 1 (12.5%) in the hybrid genotype AFLP3-cluster (Fig. 1; Table 1).

Antifungal susceptibility testing

The antifungal susceptibility profiles for amphotericin B, fluconazole and voriconazole showed that all 32 *C. neoformans* strains were sensitive to these antifungal compounds. Table 2 depicts the average of the growth inhibition zones and its distribution.

Table 2
Average growth inhibition zones.

Antifungal	Average (mm)	Minimum–maximum (mm inhibition zone)
Amphotericin B	27.55 ± 3.46	[20–33]
Fluconazole	39.06 ± 6.51	[19–48]
Voriconazole	51.48 ± 5.66	[32–59]

Discussion

The present study was performed in order to investigate the epidemiology of *C. neoformans* in a retrospective cohort of patients and from available environmental strains from Lima, Peru, using mating-type, serotype and genotype determination and antifungal susceptibility. By using AFLP genotyping, 16 (50%) of the strains were found to be genotype AFLP1 and 13 (40.6%) were genotype AFLP1B (representing *C. neoformans* var. *grubii*). Two strains (6.3%) were found to be genotype AFLP2, representing *C. neoformans* var. *neoformans*, and 1 (3.1%) was found to be genotype AFLP3 representing the hybrid between both varieties.

A previous large Ibero-Latin American epidemiological survey²⁷ showed that from the 13 clinical strains from Lima, 12 (93.3%) were genotype AFLP1 and 1 (7.7%) was AFLP4 (*C. gattii*). The same study showed that the majority of the studied Latin American *Cryptococcus* strains fell within genotype AFLP1. In Amazon-Brazil, 31 (77.5%) isolates from a total of 40 strains were identified as AFLP1 and 9 (22.5%) as genotype AFLP6 (*C. gattii*); similarly, in Goiania-Brazil,

120 (97%) of the studied isolates were AFLP1 and 4 (3%) were found to be genotype AFLP5 (*C. gattii*).^{11,29}

In Europe there was also a predominance of genotype AFLP1; however, a considerable percentage of clinical strains belong to genotype AFLP2 and AFLP3. In Madrid, Spain genotype AFLP1 represented 34 (58.9%) of the tested clinical strains.¹³ In Croatia, genotype AFLP1 represented 6 (40%) of the studied strains,²⁸ while genotype AFLP2 strains represented 11 (19%) of the studied strains in Madrid and 6 (40%) in Croatia.^{13,28} The percentage of genotype AFLP3 was found to be 21 (32.2%) in Madrid and 3 (20%) in Croatia.^{13,28} A large study performed on 300 clinical strains from the Netherlands showed that genotype AFLP1 represented 245 (81.7%) of these strains, 36 (12%) genotype AFLP2, 14 (4.7%) genotype AFLP3, 1 (0.3%) genotype AFLP4, 1 (0.3%) AFLP6 and 3 (1%) genotype AFLP8.¹⁸

Unlike what was found in Europe, the distribution of *C. neoformans* genotypes in Taiwan²⁵ is similar to what was observed in the current study, with 98 (98%) of genotype AFLP1 strains, 1 (1%) of genotype AFLP1A, and 1 (1%) AFLP4 (*C. gattii*).

Regarding antifungal susceptibility, it was observed that all the strains (clinical and environmental) were sensitive to amphotericin B, fluconazole and voriconazole. An epidemiological study in Cuba²¹ showed an antifungal sensitivity with low MICs against voriconazole and fluconazole, especially for voriconazole (MIC₉₀ of 0.064 µg/ml). A set of 120 investigated clinical and environmental samples from Goiana (Brazil) were found to be susceptible to amphotericin B, fluconazole, itraconazole and voriconazole.²⁹

In India, the antifungal susceptibility was investigated for 308 clinical and environmental strains of *C. neoformans* (AFLP1; *n* = 246, 80%) and *C. gattii* (AFLP4; *n* = 62, 20%), which showed that 306 (99.4%) of the strains were sensible to the antifungal panel (amphotericin B, flucytosine, fluconazole, itraconazole and voriconazole), and only two clinical strains of *C. neoformans* var. *grubii* were found to be resistant to flucytosine (MIC > 64 µg/ml). Nevertheless, the strains of *C. gattii* were less sensible than the strains of *C. neoformans*.⁷

In 67 clinical strains isolated from HIV-infected patients in Kenya, 63 (94%) strains of *C. neoformans* and 4 (6%) of *C. gattii* were all susceptible to amphotericin B, ravuconazole and voriconazole, and 65 (97%) to fluconazole. The remaining 2 (3%) showed dose-dependent susceptibility to fluconazole.²⁶

All these studies show that *C. neoformans* and *C. gattii* are susceptible to all the tested antifungals. These results are similar to those found in the current study.

There are very few studies in Peru on the epidemiology of *C. neoformans* and *C. gattii*. As mentioned above, an Iberoamerican study showed that the majority of the South American clinical strains belong to genotype AFLP1.²⁷ Another study investigated the presence of *C. gattii* strains among 18 clinical strains from the culture collection of the National Institute of Health from Lima, Peru, by a canavanine-glycine-bromothymol blue test. However, it was determined that all strains belonged to *C. neoformans*.⁵ Another study investigated the presence of *C. neoformans* and *C. gattii* in the environment by sampling pigeon droppings in the city of Ica, Peru, but this study did not provide a differentiation between the two pathogenic *Cryptococcus* species.⁹

Also in Peru clinical studies have been conducted in patients with cryptococcal meningitis. A study performed at the Hospital Nacional Dos de Mayo in Lima showed that the combination of amphotericin B and fluconazole leads to an acceptable rate of sterilization of the cerebrospinal fluid after 10 weeks of treatment.¹⁰ A study performed in the Hospital Cayetano Heredia in Lima showed that a low Glasgow score and a serum antigen titer greater than 1024 were associated with higher mortality.⁶ Both studies did not differentiate between the two pathogenic *Cryptococcus* species. Current evidence from Peru suggests that the majority of clinical

cases are due to *C. neoformans* var. *grubii*. However, there are clinical reports of the presence of *C. gattii* in Peru.^{4,14} One of the two Peruvian *C. gattii* strains (27) was recently found to be an inter-species hybrid between *C. neoformans* var. *neoformans* (AFLP2) and *C. gattii* (AFLP4).¹⁷

One limitation of our study is the small number of clinical samples. We have only analyzed the strains stored in our hospital culture collection. However, the study can be seen as a starting point for a national surveillance and research network. It will be interesting to carry out a nationwide prospective study to determine the presence of *C. neoformans* and *C. gattii* in clinical samples. Moreover, the environmental samples in our collection were obtained from pigeons, there were no samples obtained from soil or trees although it is known that both species can be found in these sources. Therefore, it is possible that the isolation of *C. neoformans* was favored above that of *C. gattii*. Likewise, due to the climatic conditions of Lima with low annual rainfall and a mild desert climate¹⁵ it was unlikely that the isolation of *C. gattii* in environmental samples was favored. Despite of the absence of other studies presenting a larger number of samples, we believe this work could serve as a reference for future prospective studies.

According to previous South American epidemiological studies, we noted that *C. neoformans* var. *grubii* was always the major isolated species. These studies were carried out in tropical climates, mainly in Brazil and Colombia, which may explain why the isolation of *C. gattii* was prevalent. Lima is considered a desert, with climatic features different than the major studied Latin American countries.

An interesting result was the finding of two strains of *C. neoformans* var. *neoformans*. One reason that could explain the latter is that these clinical samples came from El Callao, the main port in Peru. Moreover, our main international airport is located in the same area. The foreign traffic is heavy in this area, which could explain the presence of this genotype that is known to be more prevalent in Europe.

In conclusion, we observed that *C. neoformans* var. *grubii* (genotype AFLP1 and AFLP1B) and *C. neoformans* var. *neoformans* (genotype AFLP2) were the only cryptococcal species involved in human infections in Lima, Peru. All strains were susceptible to the antifungal compounds amphotericin B, fluconazole and voriconazole, which is consistent with previous studies.

Conflict of interest

The authors declare no conflict of interest.

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