Biomédica 2007;27:308-14

TECHNICAL NOTE

In vitro mating of Colombian isolates of the *Cryptococcus neoformans* species complex

Patricia Escandón [1], Popchai Ngamskulrungroj [2], Wieland Meyer [2], Elizabeth Castañeda [1]

[1] Grupo de Microbiología, Instituto Nacional de Salud, Bogotá, D. C., Colombia
[2] Molecular Mycology Research Laboratory, Center for Infectious Diseases and Microbiology, The University of Sydney Western Clinical School at Westmead Hospital/Westmead Millennium Institute, Westmead, NSW, Australia

Introduction. Within the *Cryptococcus neoformans* species complex, two species and five serotypes are recognized: *C. neoformans* (var. *grubii*, serotype A; var. *neoformans*, serotype D and a hybrid, serotype AD) and *C. gattii* (serotypes B and C). Mating types a and α are designated by a single locus, with the mating type α being most prevalent in serotype A and D strains.
Objective. To evaluate the ability of Colombian isolates of the *C. neoformans* species complex to mate *in vitro* with tester strains of the opposite mating type.
Materials and methods. Fifty three clinical isolates were included in this study, 33 *C. neoformans* var. *grubii* serotype A, 4 *C. neoformans* var. *neoformans* serotype D, all mating type α, and 16 *C. gattii*, 13 serotype B (mating type a) and 3 serotype C (mating type α), were mixed on V8 juice agar, using a modified method, with the appropriate tester strains to determine the mating types *in vitro*.
Results. Mating studies revealed that 9 of 33 (27.3%) serotype A isolates and 6 of 13 (46.2%) serotype B isolates were able to mate. Clamp connections and basidia with basidiospores were observed microscopically, indicating that the mating process had occurred. All mating competent serotype A strains were mating type alpha and the serotype B mating competent strains were mating type a.
Conclusion. This is the first report of the determination of the mating ability of Colombian *Cryptococcus neoformans* isolates to mate *in vitro* with appropriate tester strains, which is of great importance to study the propagation of the fungus around the globe.

Key words: *Cryptococcus neoformans*, phenotype, *in vitro*, genes, fungal

Determinación *in vitro* de la pareja sexual en aislamientos del complejo *Cryptococcus neoformans*

Introducción. En el complejo *Cryptococcus neoformans* se reconocen dos especies y cinco serotipos: *C. neoformans* (var. *grubii*, serotipo A; var. *neoformans*, serotipo D y un híbrido, serotipo AD) y *C. gattii* (serotipos B y C). La pareja sexual a y α es controlada por un solo locus, y la pareja sexual α es la más prevalente en los serotipos A y D, y es convencionalmente determinada mediante reacción en cadena de la polimerasa.
Objetivo. Evaluar la habilidad de aislamientos colombianos de *C. neoformans* para aparearse *in vitro* con aislamientos control de la pareja sexual opuesta.
Materiales y métodos. Treinta y tres aislamientos clínicos de *C. neoformans* var. *grubii* serotipo A, 4 de la var. *neoformans* serotipo D, todos pareja sexual α, y 16 aislamientos clínicos de *C. gattii*, 13 serotipo B (pareja sexual a) y 3 serotipo C (pareja sexual α), se mezclaron, en agar jugo V8 modificado, con cepas control para determinar la pareja sexual *in vitro*.
Resultados. Los estudios de apareamiento mostraron que 9 de 33 (27,3%) aislamientos serotipo A y 6 de 13 (46,2%) aislamientos serotipo B tuvieron la capacidad de aparearse con las cepas control. Todos los aislamientos del serotipo A que presentaron apareamiento eran pareja sexual α y los del serotipo B eran pareja sexual a. Microscópicamente se observaron conexiones en gancho, basidias y basidiosporas, estructuras que establecieron que se había realizado el proceso de apareamiento.

Conclusión. Este acercamiento provee por primera vez la capacidad de que los aislamientos colombianos de *C. neoformans* se apareen *in vitro* con cepas control lo cual tiene importancia en el estudio de la diseminación del hongo.

Palabras clave: *Cryptococcus neoformans*, fenotipo, *in vitro*, genes, hongos

Isolates of the *Cryptococcus neoformans* species complex cause cryptococcosis, the second most common life-threatening invasive fungal disease in humans and animals worldwide. Cryptococcosis is a systemic mycoses that affects immunocompetent and immunosuppressed individuals (1).

The *C. neoformans* species complex contains two closely related species. The first species: *C. neoformans,* which includes two varieties: *C. neoformans* var*. grubii* (serotype A, genotypes VNI/AFLP1 and VNII/AFLP1A) and *C. neoformans* var. *neoformans* (serotype D, genotype VNIV/AFLP2) as well as an AD hybrid (genotype VNIII/AFLP3), is an opportunistic pathogen that typically causes disseminated cryptococcosis in hosts with normal or impaired immunity (2-4), *C. neoformans* var*. grubii* is the major fungal pathogen in patients with AIDS (1,2). The second species: *C. gattii* (serotypes B and C, genotypes VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5 and VGIV/AFLP7) is a primary pathogen that mainly affects patients with normal immunity (3-5). Recently, a natural occurring hybrid was described between *C. neoformans* and *C. gattii* serologically characterized as BD (6). These serotypes/molecular types differ in their epidemiological, ecological, virulence and molecular characteristics (1, 4,7-11). Infections are assumed to be acquired via inhalation of infectious propagules, assumed to be desiccated yeast cells (blastoconidia) or basidiospores, from environmental niches (1,7,8).

In the *C. neoformans* species complex, the mating system is controlled by a single locus with two functional alleles, which designate the mating types *MAT*α and *MATa* (12). The mating type locus has been associated with virulence, as suggested by Kwon-Chung *et al.* (13), who found that *MAT*α strains were more virulent in a mouse model than the *MAT* a isolates. Recently it was found that the cryptococcosis outbreak on Vancouver Island, Canada was caused by isolates that were highly virulent mating type α strains belonging to the *C. gattii* molecular type VGII/AFLP6 (14,15).

Epidemiological surveys have shown that mating type α strains are most prevalent in serotype A isolates, both clinical and environmental (16), whereas in serotype B, we found that mating type a is more frequent in Colombia (17).

In our laboratory, the mating type has been determined by PCR using mating type specific primers within the *MF*1α/*MF1a* genes, which encode pheromones (16,18). The ability of *C. neoformans* strains to mate is determined by crossing the isolates with appropriate tester strains on nutrient starvation media (1,14,16). The tester strains (usually JEC 20, serotype D, mating type a, and JEC21, serotype D, mating type α) are mixed with the strains to be tested on a special media like V8 agar, SYB (sucrose, biotin, yeast extract) (19). Strains with different mating types mate to form dikaryotic hyphae, clamp connections, basidia and basidiospores, these structures are indicative of compatible mating types (20). However, the determination of the mating ability *in vitro* is associated with a number of problems, like the fact that mating is sensitive to a variety of environmental conditions, including temperature, nutrient availability, and moisture levels (1), and many strains, loose their ability to mate after being extensively manipulated (16). Despite these problems, the determination of the mating potential *in vitro* is of great interest because sexual recombination is important for the dissemination and propagation of the fungus in the environment and its transition into humans and animals (21).

The aim of the present study was to evaluate the ability of Colombian isolates of the *C. neoformans*

Correspondencia:
Patricia Escandón, Grupo de Microbiología, Instituto Nacional de Salud, Avenida calle 26 No. 51-20, Bogota, Colombia. Teléfono: 57-1-2207700 ext. 433; fax: 57-1-2207700 ext. 445
pescandon@ins.gov.co

Recibido: 15/02/07; aceptado: 30/04/07

species complex to mate *in vitro* with tester strains and produce structures associated with the mating process.

Materials and methods

Fungal isolates. Fifty three clinical isolates of *C. neoformans* and *C. gattii* recovered between 1989 and 2005 were studied. From these isolates, 33 were *C. neoformans* var. *grubii* serotype A, 4 were *C. neoformans* var. *neoformans* serotype D, 13 isolates were *C. gattii* serotype B and 3 were serotype C, as seen in table 1. The serotype was previously determined using the Crypto-Check, Iatron Laboratories, Tokyo, Japan. The mating type was previously determined by PCR, using *MAT*α and *MAT*a specific primers corresponding to the *MF1*α and *MF1a* genes (16), 40 isolates were mating type a and 13 were mating type α (table 1). All isolates originated from Colombia. The isolates were maintained as glycerol stocks at -70°C for long-term storage.

Reference strains. Strains H0058-I-1127 (JEC 20, serotype D, mating type a) and H0058-I-1128 (JEC 21, serotype D, mating type α), kindly provided by June Kwon Chung from the National Institutes of Health in Bethesda, USA, were used as tester strains for the mating (table 1).

Determination of in vitro mating. Each strain was crossed with the opposite mating partner tester strain, using a slightly modified method originally described by Kwon Chung *et al.* (22). Briefly, each strain was grown on Sabouraud agar at 27°C for 24 h. Then a tiny amount of each strain was taken using a toothpick and streak crossed each other, in a very thin layer, on V8 juice agar (5% V8 canned juice (Campbell´s), 3mM KH_2PO_4, 4% agar) adjusted to pH 5.0 with KOH 1N. Plates were incubated in darkness at 25°C for at least 4 weeks in a dry place, without wrapping the plate. Plates were examined regularly for evidence of hyphae with clamp connections, basidium, basidia and basidiospore chains indicative of mating using a microscope.

Results

Mating was observed in 15 isolates studied, from which 9 were serotype A and 6 serotype B, that mated with the opposite mating partner tester strain. As a result of the mating all mating competent serotype A strains were mating type α and the mating competent serotype B strains were mating type a, confirming the results obtained previously using PCR amplification of the *MF1α*/*MF1a* genes with *MAT*α and *MATa* specific primers.

A first sign that the mating process had occurred was the presence of filaments on the agar where the strains were mixed. Afterwards, microscopic evidence showed the presence of fungal structures (*mycelium*, clamp connections) compatible with mating *in vitro*. After conjugation had occurred, a dikaryotic *mycelium* with clamp connections was observed (figure 1A), followed by the formation of terminal basidia with basidiospores (figure 1B), which then germinate into an encapsulated yeast cell. Phenotypic differences were observed microscopically between the two species: the basidiospores of *C. neoformans* var. *grubii* were spherical or cylindrical (figure 2A), whereas those of *C. gattii* were bacilliform (figure 2B).

Discussion

The present study described the *in vitro* mating process between isolates of the *C. neoformans* species complex on V8 agar using a slightly modified protocol. The fact that each competent strain, which mating type had been previously determined by PCR, mated with the opposite tester strain, reveals a 100% concordance with the PCR. This is the first time in our laboratory, that we were able to successfully mate isolates of opposite mating types with reproducible results, since the experiment was done twice, with the same results. Several approaches had been made previously in our laboratory to test those strains, including culture on SYB agar and malt agar (22), but those experiments had been not reproducible using the previously described methodologies and the mating experiments had to be repeated more than once (23).

The sexual stage has not been conclusively identified in nature although there has been some suggestion that structures of *C. gattii* might be found in flowers from eucalyptus trees (7); however, under conditions of nitrogen starvation and relative desiccation, cells of the two mating

Table 1. *Cryptococcus neoformans/C. gattii* isolates studied and their mating type and mating abilities.

Strain #	Variety/ species	Serotype	Year of isolation	Origin	Molecular type	PCR mating type	Mating type ability
H0058-I-2374	*grubii*	A	2005	Risaralda	VNI	α	+
H0058-I-2373	*grubii*	A	2005	Norte de Santander	VNI	α	+
H0058-I-2372	*grubii*	A	2005	Cúcuta	VNI	α	-
H0058-I-2368	*grubii*	A	2005	Valle	VNI	α	-
H0058-I-2371	*grubii*	A	2005	Bogotá	VNI	α	-
H0058-I-2370	*grubii*	A	2005	Bogotá	VNI	α	+
H0058-I-2367	*grubii*	A	2005	Cúcuta	VNI	α	+
H0058-I-2364	*grubii*	A	2005	Bogotá	VNI	α	-
H0058-I-2355	*grubii*	A	2005	Cúcuta	VNI	α	-
H0058-I-2362	*grubii*	A	2005	Valle	VNI	α	-
H0058-I-2361	*grubii*	A	2005	Valle	VNII	α	-
H0058-I-2354	*grubii*	A	2005	Cúcuta	VNII	α	-
H0058-I-2350	*grubii*	A	2005	Bogotá	VNI	α	-
H0058-I-2349	*grubii*	A	2005	Antioquia	VNI	α	-
H0058-I-2348	*grubii*	A	2005	Antioquia	VNI	α	+
H0058-I-2346	*grubii*	A	2005	Cúcuta	VNI	α	+
H0058-I-2213	*grubii*	A	2004	Valle	VNI	α	-
H0058-I-2173	*grubii*	A	2004	Bogotá	VNI	α	-
H0058-I-2171	*grubii*	A	2004	Atlântico	VNII	α	-
H0058-I-2117	*grubii*	A	2004	Boyacá	VNI	α	+
H0058-I-2034	*grubii*	A	2003	Bogotá	VNI	α	+
H0058-I-2027	*grubii*	A	2003	Medellín	VNI	α	-
H0058-I-1961	*grubii*	A	2003	Bogotá	VNI	α	-
H0058-I-1934	*grubii*	A	2003	Bogotá	VNI	α	-
H0058-I-1911	*grubii*	A	2003	Bogotá	VNI	α	-
H0058-I-1909	*grubii*	A	2003	Cúcuta	VNI	α	-
H0058-I-1811	*grubii*	A	2003	Neiva	VNI	α	-
H0058-I-1788	*grubii*	A	2003	Bogotá	VNI	α	+
H0058-I-1714	*grubii*	A	2003	Valle	VNI	α	-
H0058-I-1711	*grubii*	A	2003	Bogotá	VNII	α	-
H0058-I-1651	*grubii*	A	2003	Valle	VNI	α	-
H0058-I-1812	*grubii*	A	2003	Valle	VNI	α	-
H0058-I-1276	*grubii*	A	2001	Cúcuta	VNI	α	-
H0058-I-2291	*neoformans*	D	2004	Bogotá	VNIV	α	-
H0058-I-2250	*neoformans*	D	2004	Bogotá	VNIV	α	-
H0058-I-2334	*neoformans*	D	2004	Bogotá	VNIV	α	-
H0058-I-1002	*neoformans*	D	1999	Antioquia	VNIV	α	-
H0058-I-2442	*gattii*	B	2005	Risaralda	VGIII	α	-
H0058-I-2402	*gattii*	B	2005	Casanare	VGII	a	+
H0058-I-2379	*gattii*	B	2005	Bogotá	VGII	a	-
H0058-I-2278	*gattii*	B	2004	Bogotá	VGII	a	-
H0058-I-2269	*gattii*	B	2004	Bogotá	VGII	a	-
H0058-I-2263	*gattii*	B	2004	Bogotá	VGII	a	-
H0058-I-2249	*gattii*	B	2004	Bogotá	VGII	a	-
H0058-I-2074	*gattii*	B	2003	Córdoba	VGII	a	+
H0058-I-2050	*gattii*	B	2003	Bogotá	VGII	a	+
H0058-I-2029	*gattii*	B	2003	Bogotá	VGII	a	+
H0058-I-1959	*gattii*	B	2003	Cúcuta	VGII	a	+
H0058-I-1809	*gattii*	B	2003	Bogotá	VGIII	a	-
H0058-I-1708	*gattii*	B	2003	Bogotá	VGIII	a	+
H0058-I-2086	*gattii*	C	2004	Caldas	VGIII	α	-
H0058-I-2023	*gattii*	C	2003	Caldas	VGIII	α	-
H0058-I-78	*gattii*	C	1989	Bogotá	VGIII	α	-

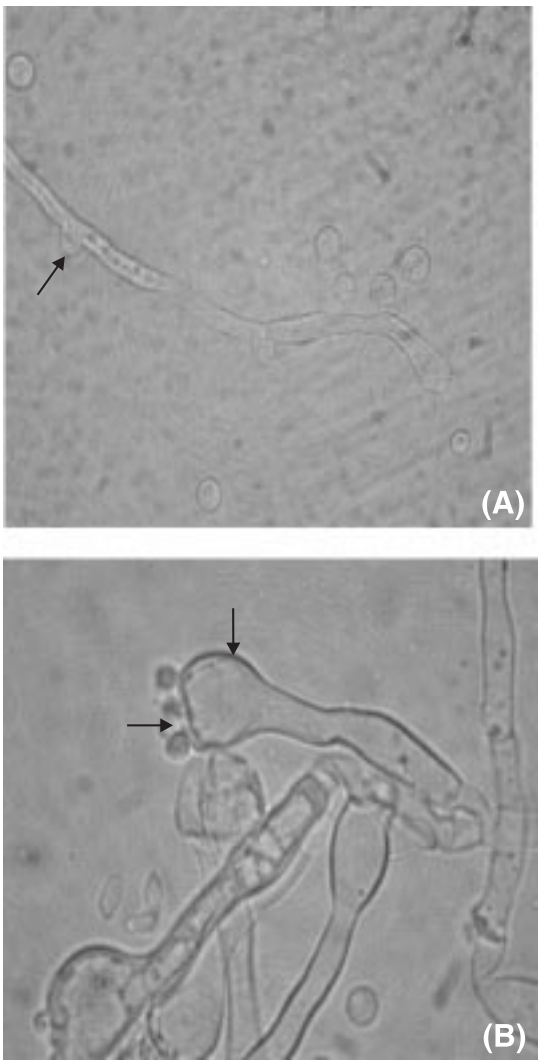

Figure 1. *Cryptococcus neoformans* structures compatible with *in vitro* mating of isolates JEC20 (*C. neoformans*, serotype D, mating type a) versus strains H0058-I-2034 (*C. neoformans* var. *grubii*, serotype A, mating type α). (A) Formation of dikaryotic mycelium with clamp connections (arrow). (B) Formation of terminal basidia with basidiospores (arrows); preparation in distilled water, 100X.

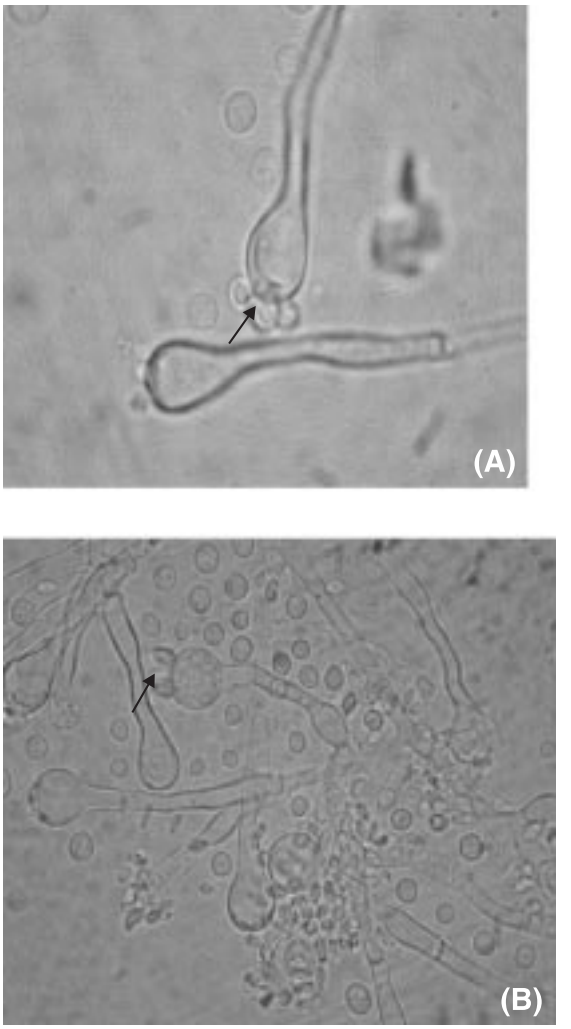

Figure 2. Phenotypic differences observed between the two species of the *Cryptococcus neoformans* complex. (A) Strain JEC20 (*C. neoformans*, serotype D, mating type a) versus strain H0058-I-2348 (*C. neoformans* var. *grubii*, serotype A, mating type α). Observe the spherical basidiospores characteristic of this specie (arrow). (B) Strain JEC21 (*C. neoformans*, serotype D, mating type α) versus strain H0058-I-2402 (*C. gattii*, serotype B, mating type a). Observe the bacilliform basidiospores characteristic of *C. gattii* (arrow); preparation in distilled water, 100X.

types in physical proximity can conjugate and form a dikaryotic mycelium in the laboratory (1) as observed in our crosses that mated successfully when culturing the isolates on V8 media using a modified method.

It is interesting to observe that the mating process occurred in isolates of both mating types in a similar ratio, 9:6 (α:a), taking into account that it had been reported, that the α mating type strains occur in over 90% of the worldwide *C. neoformans* clinical and environmental isolates (14,24). However, it was recently reported by our group that 96.6% of serotype B isolates are of mating type a (17).

It is also remarkable to show that all of our serotype B isolates, which were able to mate *in vitro,*

are of mating type a, which is interesting taking into consideration that all the isolates that produced the outbreak on Vancouver Island were of mating type α (14). The herein standardized technique will encourage us to mate the Colombian strains with those that belong to the Vancouver Island outbreak, which will help to elucidate how isolates of the *C. neoformans* species complex could have evolved into more virulent strains, which subsequently may have caused an outbreak outside the natural habitat of *C. gattii.*

Conflict of interests

The authors of the present article declare that there are no conflicts of interest that may have influenced the results of this work.

Financing

This work was supported by the Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología "Francisco José de Caldas", Colciencias (código 2104-05-14656) and the Instituto Nacional de Salud.

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